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<b>(21) International Application Number:</b> PCT/US97/09884 <b>(22) International Filing Date:</b> 6 June 1997 (06.06.97) <b>(30) Priority Data:</b> 60/020,494 11 June 1996 (11.06.96) US 9614731.9 12 July 1996 (12.07.96) GB 60/033,534 20 December 1996 (20.12.96) US  <b>(71) Applicant (for all designated States except US):</b> MERCK & CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> DONNELLY, John, J. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). LU, Tong-Ming [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). LIU, Margaret, A. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). SHIVER, John, W. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).  <b>(74) Common Representative:</b> MERCK & CO., INC.; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).		<b>(81) Designated States:</b> AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, HU, IL, IS, JP, KG, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> SYNTHETIC HEPATITIS C GENES			
<b>(57) Abstract</b>  This invention relates to novel methods and formulations of nucleic acid pharmaceutical products, specifically formulations of nucleic acid vaccine products and nucleic acid gene therapy products.			

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- 1 -

**TITLE OF THE INVENTION**  
**SYNTHETIC HEPATITIS C GENES**

**CROSS-REFERENCE TO RELATED APPLICATIONS**

5                   Not applicable.

**STATEMENT REGARDING FEDERALLY-SPONSORED R&D**  
Not applicable.

10   **REFERENCE TO MICROFICHE APPENDIX**  
Not applicable.

**FIELD OF THE INVENTION**  
Not applicable.

15   **BACKGROUND OF THE INVENTION**

          This invention relates to novel nucleic acid pharmaceutical products, specifically nucleic acid vaccine products. The nucleic acid vaccine products, when introduced directly into muscle cells, induce the production of immune responses which specifically recognize Hepatitis C virus (HCV).

**Hepatitis C Virus**

          Non-A, Non-B hepatitis (NANBH) is a transmissible disease (or family of diseases) that is believed to be virally induced, and is distinguishable from other forms of virus-associated liver disease, such as those caused by hepatitis A virus (HAV), hepatitis B virus (HBV), delta hepatitis virus (HDV), cytomegalovirus (CMV) or Epstein-Barr virus (EBV). Epidemiologic evidence suggests that there may be three types of NANBH: the water-borne epidemic type; the blood or needle associated type; and the sporadically occurring (community acquired) type. However, the number of causative agents is unknown. Recently, a new viral species, hepatitis C virus (HCV) has been identified as the primary (if not only) cause of blood-associated NANBH (BB-NANBH).

- 2 -

Hepatitis C appears to be the major form of transfusion-associated hepatitis in a number of countries, including the United States and Japan. There is also evidence implicating HCV in induction of hepatocellular carcinoma. Thus, a need exists for an effective method  
5 for preventing or treating HCV infection: currently, there is none.

The HCV may be distantly related to the flaviviridae. The Flavivirus family contains a large number of viruses which are small, enveloped pathogens of man. The morphology and composition of Flavivirus particles are known, and are discussed in M. A. Brinton, in  
10 "The Viruses: The Togaviridae And Flaviviridae" (Series eds. Fraenkel-Conrat and Wagner, vol. eds. Schlesinger and Schlesinger, Plenum Press, 1986), pp. 327-374. Generally, with respect to morphology, Flaviviruses contain a central nucleocapsid surrounded by a lipid bilayer. Virions are spherical and have a diameter of about 40-50 nm.  
15 Their cores are about 25-30 nm in diameter. Along the outer surface of the virion envelope are projections measuring about 5-10 nm in length with terminal knobs about 2 nm in diameter. Typical examples of the family include Yellow Fever virus, West Nile virus, and Dengue Fever virus. They possess positive-stranded RNA genomes (about 11,000  
20 nucleotides) that are slightly larger than that of HCV and encode a polyprotein precursor of about 3500 amino acids. Individual viral proteins are cleaved from this precursor polypeptide.

The genome of HCV appears to be single-stranded RNA containing about 10,000 nucleotides. The genome is positive-stranded,  
25 and possesses a continuous translational open reading frame (ORF) that encodes a polyprotein of about 3,000 amino acids. In the ORF, the structural proteins appear to be encoded in approximately the first quarter of the N-terminal region, with the majority of the polyprotein attributed to non-structural proteins. When compared with all known  
30 viral sequences, small but significant co-linear homologs are observed with the nonstructural proteins of the Flavivirus family, and with the pestiviruses (which are now also considered to be part of the Flavivirus family).

- 3 -

Intramuscular inoculation of polynucleotide constructs, i.e., DNA plasmids encoding proteins have been shown to result in the in situ generation of the protein in muscle cells. By using cDNA plasmids encoding viral proteins, both antibody and CTL responses were  
5 generated, providing homologous and heterologous protection against subsequent challenge with either the homologous or cross-strain protection, respectively. Each of these types of immune responses offers a potential advantage over existing vaccination strategies. The use of PNVs (polynucleotide vaccines) to generate antibodies may result  
10 in an increased duration of the antibody responses as well as the provision of an antigen that can have both the exact sequence of the clinically circulating strain of virus as well as the proper post-translational modifications and conformation of the native protein (vs. a recombinant protein). The generation of CTL responses by this means  
15 offers the benefits of cross-strain protection without the use of a live potentially pathogenic vector or attenuated virus.

Therefore, this invention contemplates methods for introducing nucleic acids into living tissue to induce expression of proteins. The invention provides a method for introducing viral  
20 proteins into the antigen processing pathway to generate virus-specific immune responses including, but not limited to, CTLs. Thus, the need for specific therapeutic agents capable of eliciting desired prophylactic immune responses against viral pathogens is met for HCV virus by this invention. Of particular importance in this therapeutic approach is the  
25 ability to induce T-cell immune responses which can prevent infections even of virus strains which are heterologous to the strain from which the antigen gene was obtained. Therefore, this invention provides DNA constructs encoding viral proteins of the hepatitis C virus core, envelope (E1), nonstructural (NS5) genes or any other HCV genes which encode  
30 products which generate specific immune responses including but not limited to CTLs.

- 4 -

DNA Vaccines

Benvenisty, N., and Reshef, L. [PNAS 83, 9551-9555, (1986)] showed that CaCl<sub>2</sub>-precipitated DNA introduced into mice intraperitoneally (i.p.), intravenously (i.v.) or intramuscularly (i.m.) could be expressed. The i.m. injection of DNA expression vectors without CaCl<sub>2</sub> treatment in mice resulted in the uptake of DNA by the muscle cells and expression of the protein encoded by the DNA. The plasmids were maintained episomally and did not replicate. Subsequently, persistent expression has been observed after i.m. injection in skeletal muscle of rats, fish and primates, and cardiac muscle of rats. The technique of using nucleic acids as therapeutic agents was reported in WO90/11092 (4 October 1990), in which polynucleotides were used to vaccinate vertebrates.

It is not necessary for the success of the method that immunization be intramuscular. The introduction of gold microprojectiles coated with DNA encoding bovine growth hormone (BGH) into the skin of mice resulted in production of anti-BGH antibodies in the mice. A jet injector has been used to transfect skin, muscle, fat, and mammary tissues of living animals. Various methods for introducing nucleic acids have been reviewed. Intravenous injection of a DNA:cationic liposome complex in mice was shown by Zhu et al., [Science 261:209-211 (9 July 1993)] to result in systemic expression of a cloned transgene. Ulmer et al., [Science 259:1745-1749, (1993)] reported on the heterologous protection against influenza virus infection by intramuscular injection of DNA encoding influenza virus proteins.

The need for specific therapeutic and prophylactic agents capable of eliciting desired immune responses against pathogens and tumor antigens is met by the instant invention. Of particular importance in this therapeutic approach is the ability to induce T-cell immune responses which can prevent infections or disease caused even by virus strains which are heterologous to the strain from which the antigen gene was obtained. This is of particular concern when dealing with HIV as this virus has been recognized to mutate rapidly and many virulent isolates have been identified [see, for example, LaRosa et al.,

- 5 -

Science 249:932-935 (1990), identifying 245 separate HIV isolates]. In response to this recognized diversity, researchers have attempted to generate CTLs based on peptide immunization. Thus, Takahashi et al., [Science 255:333-336 (1992)] reported on the induction of broadly cross-reactive cytotoxic T cells recognizing an HIV envelope (gp160) determinant. However, those workers recognized the difficulty in achieving a truly cross-reactive CTL response and suggested that there is a dichotomy between the priming or restimulation of T cells, which is very stringent, and the elicitation of effector function, including cytotoxicity, from already stimulated CTLs.

Wang et al. reported on elicitation of immune responses in mice against HIV by intramuscular inoculation with a cloned, genomic (unspliced) HIV gene. However, the level of immune responses achieved in these studies was very low. In addition, the Wang et al., DNA construct utilized an essentially genomic piece of HIV encoding contiguous Tat/REV-gp160-Tat/REV coding sequences. As is described in detail below, this is a suboptimal system for obtaining high-level expression of the gp160. It also is potentially dangerous because expression of Tat contributes to the progression of Kaposi's Sarcoma.

WO 93/17706 describes a method for vaccinating an animal against a virus, wherein carrier particles were coated with a gene construct and the coated particles are accelerated into cells of an animal.

The instant invention contemplates any of the known methods for introducing polynucleotides into living tissue to induce expression of proteins. However, this invention provides a novel immunogen for introducing proteins into the antigen processing pathway to efficiently generate specific CTLs and antibodies.

#### Codon Usage and Codon Context

The codon pairings of organisms are highly nonrandom, and differ from organism to organism. This information is used to construct and express altered or synthetic genes having desired levels of translational efficiency, to determine which regions in a genome are protein coding regions, to introduce translational pause sites into

- 6 -

heterologous genes, and to ascertain relationship or ancestral origin of nucleotide sequences

The expression of foreign heterologous genes in transformed organisms is now commonplace. A large number of mammalian genes, including, for example, murine and human genes, have been successfully inserted into single celled organisms. Standard techniques in this regard include introduction of the foreign gene to be expressed into a vector such as a plasmid or a phage and utilizing that vector to insert the gene into an organism. The native promoters for such genes are commonly replaced with strong promoters compatible with the host into which the gene is inserted. Protein sequencing machinery permits elucidation of the amino acid sequences of even minute quantities of native protein. From these amino acid sequences, DNA sequences coding for those proteins can be inferred. DNA synthesis is also a rapidly developing art, and synthetic genes corresponding to those inferred DNA sequences can be readily constructed.

Despite the burgeoning knowledge of expression systems and recombinant DNA, significant obstacles remain when one attempts to express a foreign or synthetic gene in an organism. Many native, active proteins, for example, are glycosylated in a manner different from that which occurs when they are expressed in a foreign host. For this reason, eukaryotic hosts such as yeast may be preferred to bacterial hosts for expressing many mammalian genes. The glycosylation problem is the subject of continuing research.

Another problem is more poorly understood. Often translation of a synthetic gene, even when coupled with a strong promoter, proceeds much less efficiently than would be expected. The same is frequently true of exogenous genes foreign to the expression organism. Even when the gene is transcribed in a sufficiently efficient manner that recoverable quantities of the translation product are produced, the protein is often inactive or otherwise different in properties from the native protein.



- 7 -

It is recognized that the latter problem is commonly due to differences in protein folding in various organisms. The solution to this problem has been elusive, and the mechanisms controlling protein folding are poorly understood.

5           The problems related to translational efficiency are believed to be related to codon context effects. The protein coding regions of genes in all organisms are subject to a wide variety of functional constraints, some of which depend on the requirement for encoding a properly functioning protein, as well as appropriate  
10 translational start and stop signals. However, several features of protein coding regions have been discerned which are not readily understood in terms of these constraints. Two important classes of such features are those involving codon usage and codon context.

It is known that codon utilization is highly biased and varies  
15 considerably between different organisms. Codon usage patterns have been shown to be related to the relative abundance of tRNA isoacceptors. Genes encoding proteins of high versus low abundance show differences in their codon preferences. The possibility that biases in codon usage alter peptide elongation rates has been widely discussed.  
20 While differences in codon use are associated with differences in translation rates, direct effects of codon choice on translation have been difficult to demonstrate. Other proposed constraints on codon usage patterns include maximizing the fidelity of translation and optimizing the kinetic efficiency of protein synthesis.

25           Apart from the non-random use of codons, considerable evidence has accumulated that codon/anticodon recognition is influenced by sequences outside the codon itself, a phenomenon termed "codon context." There exists a strong influence of nearby nucleotides on the efficiency of suppression of nonsense codons as well as missense codons.  
30 Clearly, the abundance of suppressor activity in natural bacterial populations, as well as the use of "termination" codons to encode selenocysteine and phosphoserine require that termination be context-dependent. Similar context effects have been shown to influence the fidelity of translation, as well as the efficiency of translation initiation.

- 8 -

Statistical analyses of protein coding regions of E. coli have demonstrate another manifestation of "codon context." The presence of a particular codon at one position strongly influences the frequency of occurrence of certain nucleotides in neighboring codons, and these  
5 context constraints differ markedly for genes expressed at high versus low levels. Although the context effect has been recognized, the predictive value of the statistical rules relating to preferred nucleotides adjacent to codons is relatively low. This has limited the utility of such nucleotide preference data for selecting codons to effect desired levels  
10 of translational efficiency.

The advent of automated nucleotide sequencing equipment has made available large quantities of sequence data for a wide variety of organisms. Understanding those data presents substantial difficulties. For example, it is important to identify the coding regions of the  
15 genome in order to relate the genetic sequence data to protein sequences. In addition, the ancestry of the genome of certain organisms is of substantial interest. It is known that genomes of some organisms are of mixed ancestry. Some sequences that are viral in origin are now stably incorporated into the genome of eukaryotic organisms. The viral  
20 sequences themselves may have originated in another substantially unrelated species. An understanding of the ancestry of a gene can be important in drawing proper analogies between related genes and their translation products in other organisms.

There is a need for a better understanding of codon context  
25 effects on translation, and for a method for determining the appropriate codons for any desired translational effect. There is also a need for a method for identifying coding regions of the genome from nucleotide sequence data. There is also a need for a method for controlling protein folding and for insuring that a foreign gene will fold appropriately  
30 when expressed in a host. Genes altered or constructed in accordance with desired translational efficiencies would be of significant worth.

Another aspect of the practice of recombinant DNA techniques for the expression by microorganisms of proteins of industrial and pharmaceutical interest is the phenomenon of "codon

- 9 -

preference". While it was earlier noted that the existing machinery for gene expression in genetically transformed host cells will "operate" to construct a given desired product, levels of expression attained in a microorganism can be subject to wide variation, depending in part on specific alternative forms of the amino acid-specifying genetic code present in an inserted exogenous gene. A "triplet" codon of four possible nucleotide bases can exist in 64 variant forms. That these forms provide the message for only 20 different amino acids (as well as transcription initiation and termination) means that some amino acids can be coded for by more than one codon. Indeed, some amino acids have as many as six "redundant", alternative codons while some others have a single, required codon. For reasons not completely understood, alternative codons are not at all uniformly present in the endogenous DNA of differing types of cells and there appears to exist a variable natural hierarchy or "preference" for certain codons in certain types of cells.

As one example, the amino acid leucine is specified by any of six DNA codons including CTA, CTC, CTG, CTT, TTA, and TTG (which correspond, respectively, to the mRNA codons, CUA, CUC, CUG, CUU, UUA and UUG). Exhaustive analysis of genome codon frequencies for microorganisms has revealed endogenous DNA of E. coli most commonly contains the CTG leucine-specifying codon, while the DNA of yeasts and slime molds most commonly includes a TTA leucine-specifying codon. In view of this hierarchy, it is generally held that the likelihood of obtaining high levels of expression of a leucine-rich polypeptide by an E. coli host will depend to some extent on the frequency of codon use. For example, a gene rich in TTA codons will in all probability be poorly expressed in E. coli, whereas a CTG rich gene will probably highly express the polypeptide. Similarly, when yeast cells are the projected transformation host cells for expression of a leucine-rich polypeptide, a preferred codon for use in an inserted DNA would be TTA.

The implications of codon preference phenomena on recombinant DNA techniques are manifest, and the phenomenon may

- 10 -

serve to explain many prior failures to achieve high expression levels of exogenous genes in successfully transformed host organisms—a less "preferred" codon may be repeatedly present in the inserted gene and the host cell machinery for expression may not operate as efficiently.

- 5 This phenomenon suggests that synthetic genes which have been designed to include a projected host cell's preferred codons provide a preferred form of foreign genetic material for practice of recombinant DNA techniques.

10 Protein Trafficking

- The diversity of function that typifies eukaryotic cells depends upon the structural differentiation of their membrane boundaries. To generate and maintain these structures, proteins must be transported from their site of synthesis in the endoplasmic reticulum to  
15 predetermined destinations throughout the cell. This requires that the trafficking proteins display sorting signals that are recognized by the molecular machinery responsible for route selection located at the access points to the main trafficking pathways. Sorting decisions for most proteins need to be made only once as they traverse their  
20 biosynthetic pathways since their final destination, the cellular location at which they perform their function, becomes their permanent residence.

- Maintenance of intracellular integrity depends in part on the selective sorting and accurate transport of proteins to their correct  
25 destinations. Over the past few years the dissection of the molecular machinery for targeting and localization of proteins has been studied vigorously. Defined sequence motifs have been identified on proteins which can act as 'address labels'. A number of sorting signals have been found associated with the cytoplasmic domains of membrane proteins.

30

**SUMMARY OF THE INVENTION**

This invention relates to novel formulations of nucleic acid pharmaceutical products, specifically nucleic acid vaccine products. The nucleic acid products, when introduced directly into muscle cells,

- 11 -

induce the production of immune responses which specifically recognize Hepatitis C virus (HCV).

#### BRIEF DESCRIPTION OF THE DRAWINGS

- 5                   Figure 1 shows the nucleotide sequence of the V1Ra vector.  
                  Figure 2 is a diagram of the V1Ra vector.  
                  Figure 3 is a diagram of the Vtpa vector.  
                  Figure 4 is the VUb vector  
                  Figure 5 shows an optimized sequence of the HCV core  
10   antigen.  
                  Figure 6 shows V1Ra.HCV1CorePAb, Vtpa.HCV1CorePAb  
                  and VUb.HCV1CorePAb.  
                  Figure 7 shows the Hepatitis C Virus Core Antigen  
                  Sequence.  
15                   Figure 8 shows codon utilization in human protein-coding  
                  sequences (from Lathe et al.).  
                  Figure 9 shows an optimized sequence of the HCV E1  
                  protein.  
                  Figure 10 shows an optimized sequence of the HCV E2  
20   protein.  
                  Figure 11 shows an optimized sequence of the HCV E1 +E2  
                  proteins.  
                  Figure 12 shows an optimized sequence of the HCV NS5a  
                  protein.  
25                   Figure 13 shows an optimized sequence of the HCV NS5b  
                  protein.

#### DETAILED DESCRIPTION OF THE INVENTION

- 30                   This invention relates to novel formulations of nucleic acid  
                  pharmaceutical products, specifically nucleic acid vaccine products.  
                  The nucleic acid vaccine products, when introduced directly into muscle  
                  cells, induce the production of immune responses which specifically  
                  recognize Hepatitis C virus (HCV).

- 12 -

Non-A, Non-B hepatitis (NANBH) is a transmissible disease (or family of diseases) that is believed to be virally induced, and is distinguishable from other forms of virus-associated liver disease, such as those caused by hepatitis A virus (HAV), hepatitis B virus (HBV),  
5 delta hepatitis virus (HDV), cytomegalovirus (CMV) or Epstein-Barr virus (EBV). Epidemiologic evidence suggests that there may be three types of NANBH: the water-borne epidemic type; the blood or needle associated type; and the sporadically occurring (community acquired) type. However, the number of causative agents is unknown. Recently, a  
10 new viral species, hepatitis C virus (HCV) has been identified as the primary (if not only) cause of blood-associated NANBH (BB-NANBH). Hepatitis C appears to be the major form of transfusion-associated hepatitis in a number of countries, including the United States and Japan. There is also evidence implicating HCV in induction of  
15 hepatocellular carcinoma. Thus, a need exists for an effective method for preventing or treating HCV infection: currently, there is none.

The HCV may be distantly related to the flaviviridae. The Flavivirus family contains a large number of viruses which are small, enveloped pathogens of man. The morphology and composition of  
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25 bilayer. Virions are spherical and have a diameter of about 40-50 nm. Their cores are about 25-30 nm in diameter. Along the outer surface of the virion envelope are projections measuring about 5-10 nm in length with terminal knobs about 2 nm in diameter. Typical examples of the family include Yellow Fever virus, West Nile virus, and Dengue Fever  
30 virus. They possess positive-stranded RNA genomes (about 11,000 nucleotides) that are slightly larger than that of HCV and encode a polyprotein precursor of about 3500 amino acids. Individual viral proteins are cleaved from this precursor polypeptide.

- 13 -

The genome of HCV appears to be single-stranded RNA containing about 10,000 nucleotides. The genome is positive-stranded, and possesses a continuous translational open reading frame (ORF) that encodes a polyprotein of about 3,000 amino acids. In the ORF, the structural proteins appear to be encoded in approximately the first quarter of the N-terminal region, with the majority of the polyprotein attributed to non-structural proteins. When compared with all known viral sequences, small but significant co-linear homologics are observed with the nonstructural proteins of the Flavivirus family, and with the pestiviruses (which are now also considered to be part of the Flavivirus family).

Intramuscular inoculation of polynucleotide constructs, i.e., DNA plasmids encoding proteins have been shown to result in the generation of the encoded protein in situ in muscle cells. By using cDNA plasmids encoding viral proteins, both antibody and CTL responses were generated, providing homologous and heterologous protection against subsequent challenge with either the homologous or cross-strain protection, respectively. Each of these types of immune responses offers a potential advantage over existing vaccination strategies. The use of PNVs (polynucleotide vaccines) to generate antibodies may result in an increased duration of the antibody responses as well as the provision of an antigen that can have both the exact sequence of the clinically circulating strain of virus as well as the proper post-translational modifications and conformation of the native protein (vs. a recombinant protein). The generation of CTL responses by this means offers the benefits of cross-strain protection without the use of a live potentially pathogenic vector or attenuated virus.

The standard techniques of molecular biology for preparing and purifying DNA constructs enable the preparation of DNA therapeutics of this invention. While standard techniques of molecular biology are therefore sufficient for the production of the products of this invention, the specific constructs disclosed herein provide novel therapeutics which surprisingly produce cross-strain

- 14 -

protection, a result heretofore unattainable with standard inactivated whole virus or subunit protein vaccines.

The amount of expressible DNA to be introduced to a vaccine recipient will depend on the strength of the transcriptional and translational promoters used in the DNA construct, and on the immunogenicity of the expressed gene product. In general, an immunologically or prophylactically effective dose of about 1  $\mu$ g to 1 mg, and preferably about 10  $\mu$ g to 300  $\mu$ g is administered directly into muscle tissue. Subcutaneous injection, intradermal introduction, impression through the skin, and other modes of administration such as intraperitoneal, intravenous, or inhalation delivery are also contemplated. It is also contemplated that booster vaccinations are to be provided.

The DNA may be naked, that is, unassociated with any proteins, adjuvants or other agents which impact on the recipient's immune system. In this case, it is desirable for the DNA to be in a physiologically acceptable solution, such as, but not limited to, sterile saline or sterile buffered saline. Alternatively, the DNA may be associated with surfactants, liposomes, such as lecithin liposomes or other liposomes known in the art, as a DNA-liposome mixture, (see for example WO93/24640) or the DNA may be associated with an adjuvant known in the art to boost immune responses, such as a protein or other carrier. Agents which assist in the cellular uptake of DNA, such as, but not limited to, calcium ions, detergents, viral proteins and other transfection facilitating agents may also be used to advantage. These agents are generally referred to as transfection facilitating agents and as pharmaceutically acceptable carriers. As used herein, the term gene refers to a segment of nucleic acid which encodes a discrete polypeptide. The term pharmaceutical, and vaccine are used interchangeably to indicate compositions useful for inducing immune responses. The terms construct, and plasmid are used interchangeably. The term vector is used to indicate a DNA into which genes may be cloned for use according to the method of this invention.



- 15 -

The following examples are provided to further define the invention, without limiting the invention to the specifics of the examples.

5

### EXAMPLE 1

#### VIIJ EXPRESSION VECTORS:

VIIJ is derived from vectors VI and pUC18, a commercially available plasmid. VI was digested with SspI and EcoRI restriction enzymes producing two fragments of DNA. The smaller of these fragments, containing the CMVintA promoter and Bovine Growth Hormone (BGH) transcription termination elements which control the expression of heterologous genes, was purified from an agarose electrophoresis gel. The ends of this DNA fragment were then "blunted" using the T4 DNA polymerase enzyme in order to facilitate its ligation to another "blunt-ended" DNA fragment.

pUC18 was chosen to provide the "backbone" of the expression vector. It is known to produce high yields of plasmid, is well-characterized by sequence and function, and is of minimum size. We removed the entire *lac* operon from this vector, which was unnecessary for our purposes and may be detrimental to plasmid yields and heterologous gene expression, by partial digestion with the HaeII restriction enzyme. The remaining plasmid was purified from an agarose electrophoresis gel, blunt-ended with the T4 DNA polymerase, treated with calf intestinal alkaline phosphatase, and ligated to the CMVintA/BGH element described above. Plasmids exhibiting either of two possible orientations of the promoter elements within the pUC backbone were obtained. One of these plasmids gave much higher yields of DNA in *E. coli* and was designated VIIJ. This vector's structure was verified by sequence analysis of the junction regions and was subsequently demonstrated to give comparable or higher expression of heterologous genes compared with VI. The ampicillin resistance marker was replaced with the neomycin resistance marker to yield vector VIIJneo.

- 16 -

An Sfi I site was added to VIJneo to facilitate integration studies. A commercially available 13 base pair Sfi I linker (New England BioLabs) was added at the Kpn I site within the BGH sequence of the vector. VIJneo was linearized with Kpn I, gel purified, blunted by T4 DNA polymerase, and ligated to the blunt Sfi I linker. Clonal isolates were chosen by restriction mapping and verified by sequencing through the linker. The new vector was designated VIJns. Expression of heterologous genes in VIJns (with Sfi I) was comparable to expression of the same genes in VIJneo (with Kpn I).

Vector VIRa (Sequence is shown in Figure 1; map is shown in Figure 2) was derived from vector VIR, a derivative of the VIJns vector. Multiple cloning sites (*Bgl*II, *Kpn*I, *Eco*RV, *Eco*RI, *Sall*, and *Not*I) were introduced into VIR to create the VIRa vector to improve the convenience of subcloning. VIRa vector derivatives containing the tpa leader sequence and ubiquitin sequence were generated (Vtpa (Figure 3) and Vub (Figure 4), respectively). Expression of viral antigen from Vtpa vector will target the antigen protein into the exocytic pathway, thus producing a secretable form of the antigen proteins. These secreted proteins are likely to be captured by professional antigen presenting cells, such as macrophages and dendritic cells, and processed and presented by class II molecules to activate CD4<sup>+</sup> Th cells. They also are more likely to efficiently simulate antibody responses. Expression of viral antigen through VUb vector will produce a ubiquitin and antigen fusion protein. The uncleavable ubiquitin segment (glycine to alanine change at the cleavage site, Butt et al., JBC 263:16364, 1988) will target the viral antigen to ubiquitin-associated proteasomes for rapid degradation. The resulting peptide fragments will be transported into the ER for antigen presentation by class I molecules. This modification is attempted to enhance the class I molecule-restricted CTL responses against the viral antigen (Townsend et al, JEM 168:1211, 1988).

- 17 -

**EXAMPLE 2****DESIGN AND CONSTRUCTION OF THE SYNTHETIC GENES****A. Design of Synthetic Gene Segments for HCV Gene Expression:**

- 5           Gene segments were converted to sequences having identical translated sequences (except where noted) but with alternative codon usage as defined by R. Lathe in a research article from *J. Molec. Biol.* Vol. 183, pp. 1-12 (1985) entitled "Synthetic Oligonucleotide Probes Deduced from Amino Acid Sequence Data: Theoretical and
- 10   Practical Considerations". The methodology described below was based on our hypothesis that the known inability to express a gene efficiently in mammalian cells is a consequence of the overall transcript composition. Thus, using alternative codons encoding the same protein sequence may remove the constraints on HCV gene expression.
- 15   Inspection of the codon usage within HCV genome revealed that a high percentage of codons were among those infrequently used by highly expressed human genes. The specific codon replacement method employed may be described as follows employing data from Lathe et al.:
- 20           1.   Identify placement of codons for proper open reading frame.
2.   Compare wild type codon for observed frequency of use by human genes (refer to Table 3 in Lathe et al.).
3.   If codon is not the most commonly employed,
- 25   replace it with an optimal codon for high expression based on data in Table 5.
4.   Inspect the third nucleotide of the new codon and the first nucleotide of the adjacent codon immediately 3'- of the first. If a 5'-CG-3' pairing has been created by the new codon selection, replace it
- 30   with the choice indicated in Table 5.
5.   Repeat this procedure until the entire gene segment has been replaced.
6.   Inspect new gene sequence for undesired sequences generated by these codon replacements (e.g., "ATTTA" sequences,

- 18 -

inadvertent creation of intron splice recognition sites, unwanted restriction enzyme sites, etc.) and substitute codons that eliminate these sequences.

7. Assemble synthetic gene segments and test for improved expression.

#### B. HCV CORE ANTIGEN SEQUENCE

The consensus core sequence of HCV was adopted from a generalized core sequence reported by Bukh et al. (PNAS, 91:8239, 1994). This core sequence contains all the identified CTL epitopes in both human and mouse. The gene is composed of 573 nucleotides and encodes 191 amino acids. The predicted molecular weight is about 23 kDa.

The codon replacement was conducted to eliminate codons which may hinder the expression of the HCV core protein in transfected mammalian cells in order to maximize the translational efficiency of DNA vaccine. Twenty three point two percent (23.2%) of nucleotide sequence (133 out of 573 nucleotides) were altered, resulting in changes of 61.3% of the codons (117 out 191 codons) in the core antigen sequence. The optimized nucleotide sequence of HCV core is shown in Figure 5.

#### C. CONSTRUCTION OF THE SYNTHETIC CORE GENE

The optimized HCV core gene (Figure 5) was constructed as a synthetic gene annealed from multiple synthetic oligonucleotides. To facilitate the identification and evaluation of the synthetic gene expression in cell culture and its immunogenicity in mice, a CTL epitope derived from influenza virus nucleoprotein residues 366-374 and an antibody epitope sequence derived from SV40 T antigen residues 684-698 were tagged to the carboxyl terminal of the core sequence (Figure 6). For clinical use it may be desired to express the core sequence without the nucleoprotein 366-374 and SV40 T 684-698 sequences. For this reason, the sequence of the two epitopes is flanked by two *EcoRI* sites which will be used to excise this fragment of

- 19 -

sequence at a later time. Thus an embodiment of the invention for clinical use could consist of the VIRa.HCV1CorePAb, Vtpa.HCV1CorePAb, or VUb.HCV1CorePAb plasmids that had been cut with EcoRI, annealed, and ligated to yield plasmids

5 VIRa.HCV1Core, Vtpa.HCV1Core, and VUb.HCV1Core.

The synthetic gene was built as three separate segments in three vectors, nucleotides 1 to 80 in VIRa, nucleotides 80 to 347 (*Bst*XI site) in pUC18, and nucleotides 347 to 573 plus the two epitope sequence in pUC18. All the segments were verified by DNA  
10 sequencing, and joined together in VIRa vector.

#### D. HCV Gene Expression Constructs:

In each case, the junction sequences from the 5' promoter region (CMVintA) into the cloned gene is shown. The position at which  
15 the junction occurs is demarcated by a "/", which does not represent any discontinuity in the sequence.

The nomenclature for these constructs follows the convention: "Vector name-HCV strain-gene".

20

#### **VIRa.HCV1.CorePAb**

---IntA--AGA TCT ACC / ATG AGC--HCV.Core--GCC / GAA TTC GCT TCC--  
PAb Sequence--TAA / ACC CGG GAA TTC TAA A / GTC GAC--BGH---

25

#### **Vtpa.HCV1.CorePAb**

--IntA--ATC ACC / ATG GAT--tpa leader--GAG ATC-TTC / ATG AGC--  
HCV.Core--GCC / GAA TTC GCT TCC--PAb Sequence--TAA / ACC CGG GAA  
TTC TAA A / GTC GAC--BGH---

30

#### **VUb.HCV1.CorePAb.**

--IntA--AGA TCC ACC / ATG CAG--Ubiquitin--GGT GCA GAT CTG/ ATG AGC--  
HCV.Core--GCC / GAA TTC GCT TCC--PAb Sequence--TAA / ACC CGG GAA  
TTC TAA A / GTC GAC--BGH---

- 20 -

**VIRa.HCV1.Core**

--IntA--AGA TCT ACC / ATG AGC--HCV.Core--GCC / TAA A / GTC GAC--  
BGH---

**5                   Vtpa.HCV1.Core**

--IntA--ATC ACC / ATG GAT--tpa leader--GAG ATC-TTC / ATG AGC--  
HCV.Core--GCC / TAA A / GTC GAC--BGH---

**VUb.HCV1.Core**

**10**--IntA--AGA TCC ACC / ATG CAG--Ubiquitin--GGT GCA GAT CTG/ ATG AGC--  
HCV.Core--GCC / TAA A / GTC GAC--BGH---

**E. OTHER SYNTHETIC HCV GENES**

**15**Using similar codon optimization techniques, synthetic  
genes encoding the HCV E1 (Figure 9), HCV E2 (Figure 10), HCV  
E1+E2 (Figure 11), HCV NS5a (Figure 12) and HCV NS5b (Figure 13)  
proteins were created.

- 21 -

## WHAT IS CLAIMED:

1. A synthetic polynucleotide comprising a DNA sequence encoding an HCV protein selected from the group consisting of HCV core protein, HCV E1 protein, HCV E1+E2 protein, HCV NS5a protein, HCV NS5b protein and fragments thereof, the DNA sequence comprising codons optimized for expression in a vertebrate host.
2. A plasmid vector comprising the polynucleotide of Claim 1, the plasmid vector being suitable for immunization of a vertebrate host.
3. The polynucleotide of Claim 1 which is HCV genotype I/1a core.

## 4. The polynucleotide of Claim 1 having the sequence

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1 ATGAGTACCA AGCTCAAGTC CTAGATGAAG ATCAAGAGCA AATGTAAGC GAGTCCCTAC CATCTGAAGT TTCTGGGG 80
81 AGAGTACATC GTTGGAGAGG TTACCTCTCT CTGAGAGACT GGTGTAAGC TGGAGTTAGG GGTAGAGAGT AAGATATG 160
161 AAGATATGCA GTTGGAGAGG AGTGGAGACT CTATGTCGAA GGTGTAAGC CTGAGAGAGG GTTGTGCTCT CAGATATG 240
241 TATGCTCTCT GTTGTGCTCT CAGATGAAGC TTGCGCTGCG GGTGTAAGC GTTGTGCTCT GGTGTAAGC GGTGTAAGC 320
321 GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC 400
401 TGGCTGATAT GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC 480
481 GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC 560
561 GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC 571

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## 5. The plasmid vector of Claim 2 having the sequence

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1 CATATGCTCT ATGAGTACAT CTAGATGAAG TATGATATAT ATATATATTA CATTTATATT GGTGTAAGC CAGATATG 80
81 CATATGCTCT CATTTATATT TACTATATTA TATGATATTA TATGATATTA CATTTATATT GGTGTAAGC CAGATATG 160
161 TGGCTGCTCT ATATGATTAAG GTAGATGAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC GGTGTAAGC 240
241 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 320
321 ATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 400
401 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 480
481 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 560
561 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 640
641 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 720
721 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 800
801 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 880
881 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 960
961 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 1040
1041 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 1120
1121 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 1200
1201 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 1280
1281 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 1360
1361 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 1440
1441 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 1520
1521 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 1600
1601 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 1680
1681 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 1760
1761 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 1840
1841 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 1920
1921 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 2000
2001 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 2080
2081 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 2160
2161 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 2240
2241 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 2320
2321 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 2400
2401 TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA TATGATATTA 2480

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- 22 -

2481 GTATTCTAGG GGGTGGGTTG GGGGAGGAGA GCAAGGGGGA GGAATTGGAA GAGCAATAGCA GGAATGTG GGAATGAGTT 2560  
 2482 GATTTATATG GTATGAGGTT AGGGGTTTA ATTAAAGGAG GAGGGGGTTT AGTCAAGTGG TGAGCAATTT ACTTGTGTTT 2640  
 2483 TGGAGTTTGA AAAAGGGTGG GTTGTGGTGG TTTTTCATA GGGTGGGTTT GGTGGAGTGG CATACAAAAA ATTTGAGTTT 2720  
 2721 AAGTCAAGAG TGGGAGAAAG GAGTCAAGAT ATAAAGATAG GAGGGTTTTC GGTGGAGTGG GTTGTGGTGG GGTGTGGTGG 2800  
 2801 TTTTGGGTTT GGGGTTTACG GATAGCTGCT GGGGTTTCT GGTGTGGTGG AGGGGGTGG TTTGTGAATG GTTAAAGTGT 2880  
 2881 AGGATGTGTA GTTGTGTGTA GGTGTGGTGG TGAAGGTTGG GGTGTGGTGG GAGAGTGGT GGAAGTGGT AGTCAAGTGT 2960  
 2961 GTTATGAGT AACTATGCTG TGAAGTGA GGGGTTAGGA GAGCAATTTAT GGTCACTGGT AGTCAAGTGT GTTAAAGTGG 3040  
 3041 TTAGCAAGAG GAGTATGTA GGGGTTGTA GAGAGTGT TT GAGTGTGG GTTAACTAGG GTTCACTAGG AAGTCAAGT 3120  
 3121 TTTGTATGTT GGTGTGTGTT GAGGTTAGTT AATTGTGAAA AAGAGTGGT TAGGTGTGA TGGGTAAAT AAGTCAAGT 3200  
 3201 TTTGTGGTGT GGTGTGTGTT TTTGTAAACA GAGATTTAG GGTGTGGTGG AAGTCAAGT AAGTCAAGT AAGATGTTT TGTATTTTTT 3280  
 3281 GTAGTGGTGT GGTGTGTGTT GTTGTAGTGT TAAAGTAAAT TAAGCAATTT TGTATTGAAA AATGTATGTA GTTAAAGT 3360  
 3361 AAAGTCAAT TTATTCATAT GAGATTTAT AATCAATAT TTTTAAAAAA GGTGTGTGTT TAATTGAGTA GAGAAATGAC 3440  
 3441 GAGAGTGTGT GTTAAAGTGG GAGATTTAT GTTGTGGTGT TGTGTATGTT AATTGTGAAA CATCAATGTA AATTTATTAAT 3520  
 3521 TTTGTGGTGT GAGAAATAGG GTTATTAAGT GAGAAATCA GATCAATGAG GATCAATGCT GTTCAAGTGT GAGAAAGTTT 3600  
 3601 ATGTATTGTT TTTCAAGTT GTTAAAGTGG GAGAAATTTA GGTGTGTGAT GAGAAATGAT GTTATTAAGT AAGTGTATAT 3680  
 3681 TATTATGTA TTTGTGTA GTTAAAGTGG ATRAGTGTGT GGTGTGTGTA GAGCAATTTA AAGTGTGAT GGAATGTAAT 3760  
 3761 GGTGTGTA AGAGTGTGG GTTAAAGTGG ATATTGTA ATRAGTGTGT GTTAAAGTGG ATATTGTT AATATGTA ATRAGTGTGT 3840  
 3841 GGTGTGTA AGAGTGTGG GTTAAAGTGG ATATTGTA ATRAGTGTGT GTTAAAGTGG ATRAGTGTGT ATRAGTGTGT 3920  
 3921 GGTGTGTA AGAGTGTGG GTTAAAGTGG ATATTGTA ATRAGTGTGT GTTAAAGTGG ATRAGTGTGT ATRAGTGTGT 4000  
 4001 GGTGTGTA AGAGTGTGG GTTAAAGTGG ATATTGTA ATRAGTGTGT GTTAAAGTGG ATRAGTGTGT ATRAGTGTGT 4080  
 4081 TAAATGTA TTTGTGTT AATTGATTT GGTGTGTA GGTGTGTT GAGATTTT GAGATTTT TATATGTA 4160  
 4161 TATTATGTT TATTATGTA GAGATTTT TTTGTGTA TATATGTT TATATGTT TATATGTT TATATGTA 4240  
 4241 TATATGTA GAGATTTT C

25           6. The polynucleotide of Claim 4 from which the PAb sequence has been removed.

30           7. The plasmid vector of Claim 5 from which the PAb sequence has been removed.

35           8. A method for inducing immune responses in a vertebrate against HCV epitopes which comprises introducing between 1 ng and 100 mg of the polynucleotide of Claim 1 into the tissue of the vertebrate.

          9. A method for inducing immune responses against infection or disease caused by HCV which comprises introducing into the tissue of a vertebrate the polynucleotide of Claim 1.

40           10. A vaccine for inducing immune responses against HCV infection which comprises the polynucleotide of Claim 1 and a pharmaceutically acceptable carrier.

45           11. A method for inducing anti-HCV immune responses in a primate which comprises introducing the polynucleotide of Claim 1 into the tissue of said primate and concurrently administering interleukin-12 parenterally.



- 23 -

12. A method of inducing an antigen presenting cell to stimulate cytotoxic and helper T-cell proliferation and effector functions including lymphokine secretion specific to HCV antigens which  
5 comprises exposing cells of a vertebrate in vivo to the polynucleotide of Claim 1.

13. A method of treating a patient in need of such treatment comprising administering to the patient the polynucleotide of  
10 Claim 1 in combination with interferon-alpha, Ribavirin, Zidovudine, or other pharmaceutically acceptable antiviral agents..

14. A pharmaceutical composition comprising the polynucleotide of Claim 1.  
15

15. A method of inducing an immune response comprising administering the polynucleotide of Claim 1 to a patient, the  
administration of the polynucleotide antedating or coinciding or  
following administration to the patient of a subunit, recombinant,  
20 recombinant live vector, inactivated, recombinant inactivated vector, or live attenuated HCV vaccine.

16. A method for inducing immune responses in a vertebrate against HCV epitopes which comprises introducing between 1  
25 ng and 100 mg of the polynucleotide of Claim 2 into the tissue of the vertebrate.

17. A method for inducing immune responses against infection or disease caused by HCV which comprises introducing into  
30 the tissue of a vertebrate the polynucleotide of Claim 2.

18. A vaccine for inducing immune responses against HCV infection which comprises the polynucleotide of Claim 2 and a pharmaceutically acceptable carrier.

- 24 -

19. A method for inducing anti-HCV immune responses in a primate which comprises introducing the polynucleotide of Claim 2 into the tissue of said primate and concurrently administering  
5 interleukin 12 parenterally.

20. A method of inducing an antigen presenting cell to stimulate cytotoxic and helper T-cell proliferation and effector functions including lymphokine secretion specific to HCV antigens which  
10 comprises exposing cells of a vertebrate in vivo to the polynucleotide of Claim 2.

21. A method of treating a patient in need of such treatment comprising administering to the patient the polynucleotide of  
15 Claim 2 in combination with interferon-alpha, Ribavirin, Zidovudine, or other pharmaceutically acceptable antiviral agents..

22. A pharmaceutical composition comprising the  
20 polynucleotide of Claim 2.

23. A method of inducing an immune response comprising administering the polynucleotide of Claim 2 to a patient, the administration of the polynucleotide antedating or coinciding or following administration to the patient of a subunit, recombinant,  
25 recombinant live vector, inactivated, recombinant inactivated vector, or live attenuated HCV vaccine.

24. The vector of Claim 2 which is selected from  
VIRa.HCV1CorePAb, Vtpa.HCV1CorePAb, VUb.HCV1CorePAb,  
30 VIRa.HCV1Core, Vtpa.HCV1Core and VUb.HCV1Core.

25. A pharmaceutical composition comprising the vector of Claim 21.

- 25 -

26. The DNA sequence of Claim 1 selected from the group consisting of a nucleotide sequence shown in Figure 5, Figure 9, Figure 10, Figure 11, Figure 12 and Figure 13.

	10	20	30	40	50	60	70	80
1	GATATTGGCT	ATTGGCCATT	GCATAGCTTG	TATCCATATC	ATAATATGTA	CATTATAT	GGCICATGTC	CAACATTACC
81	GCCATGTTGA	CATTGATTAT	TGACTAGTTA	TTAATAGTAA	TCAATTACGG	GGTCATTAGT	TCATAGCCCA	TATATGGAGT
161	TCCGGTTAC	ATAACTTACG	GTAATGGCC	CGCTGGCTG	ACCGCCCAAC	GACCCCGCC	CATTGACGTC	AATAATGACG
241	TATGTTCCCA	TAGTAACGCC	AATAGGGACT	TTCCATTGAC	GTCAATGGGT	GGAGTATTTA	CGGTAAACTG	CCCACTTGGC
321	AGTACATCAA	GTGTATCATA	TGCCAAGTAC	GCCCCCTATT	GACGTCAATG	ACGGTAAATG	GCCCCGCTGG	CATTATGCCC
401	AGTACATGAC	CTTATGGGAC	TTTCCTACTT	GGCAGTACAT	CTACGTATTA	GTCATCGCTA	TTACCATGGT	GATCGGGTTT
481	TGGCAGTACA	TCAATGGGCG	TGGATAGCGG	TTTGACTCAC	GGGGATTTC	AAGTCTCCAC	CCCATTGACG	TCAATGGGAG
561	TTTGTTTTGG	CACCAAATC	AACGGGACTT	TCCAAAATGT	CGTAAACACT	CCGCCCCATT	GACGCAAAATG	GGCGGTAGGC
641	GTGTACGGTG	GGAGGTCTAT	ATAAGCAGAG	CTCGTTTAGT	GAACCGTCAG	ATCGCCTGGA	GACGCCATCC	ACGCTGTTTT
721	GACCTCCATA	GAAGACACCG	GGACCGATCC	AGCCTCGCG	GCCGGGAACG	GTGCATTGGA	ACGCGGATTC	CCCGTGCCAA
801	GAGTGACGTA	AGTACCGCCT	ATAGAGTCTA	TAGGCCACC	CCCTTGGCTT	CTTATGCGATG	CTATACTGTT	TTTGGCTTGG
881	GGTCTATACA	CCCCCGCTC	CTCATGTTAT	AGGTGATGGT	ATAGCTTAGC	CTATAGGTGT	GGGTTATTGA	CCATTATTGA
961	CCACTCCCCT	ATTGGTGACG	ATACTTTCCA	TTACTAATCC	ATAACATGGC	TCTTTGCCAC	AACTCTCTTT	ATTGGCTATA
1041	TGCCAATACA	CTGTCCCTCA	GAGACTGACA	CGGACTCTGT	ATTTTTACAG	GATGGGGTCT	CATTTATTAT	TTACAAATTC
1121	ACATATACAA	CACCACCGTC	CCCAGTGCCC	GCAGTTTITA	TTAAACATAA	CGTGGGATCT	CCACGCGAAT	CTCGGGTACG
1201	TGTTCCGGAC	ATGGGCTCTT	CTCCGGTAGC	GGCGGAGCTT	CTACATCCGA	GCCCTGCTCC	CATGCTCCA	GCGACTCATG
1281	GTGCTCGGC	AGCTCCTTGC	TCCTAACAGT	GGAGGCCAGA	CTTAGGCACA	GCAGATGCC	CACCACCACC	AGTGTGCCGC
1361	ACAAGGCCGT	GGCGGTAGGG	TATGTGTCTG	AAAATGAGCT	CGGGGAGCGG	GCTTGCACCG	CTGACGCAAT	TGGAAGACTT
1441	AAGGCAGCGG	CAGAAGAAGA	TGCAGGCAGC	TGAGTTGTTG	TGTTCTGATA	AGAGTCAGAG	GTAACCTCCG	TTGCGGTGCT
1521	GTTAACGGTG	GAGGGCAGTG	TAGTCTGAGC	AGTACTCGTT	GCTGCGCGC	GCGCCACCAG	ACATAATAGC	TGACAGACTA
1601	ACAGACTGTT	CCTTTCCATG	GGCTTTTCT	GCAGTCACCG	TCCTTAGATC	TAGGTACCAG	ATATCAGAA	TCAGTCGACA
1680	GCGGCCCGGA	TCTGCTGTGC	CTTCTAGTTG	CCAGCCATCT	GTTGTTTGCC	CCTCCCCCGT	GCCTTCTCTG	ACCCTGGAAG
1761	GTGCCACTCC	CAGTGTCTT	TCCTAATAAA	ATGAGGAAAT	TGCATCGCAT	TGTTCTGAGTA	GGTGTCAATC	TATTTCTGGG
1841	GCTGGGGTGG	GGCAGCACAG	CAAGGGGGAG	GATTGGSAAG	ACAATAGCAG	GCAATGCTGG	GATCGGGTGG	GCTCTATGGG
1921	TACGGCCCGCA	GCGGCCTTAA	TTAAGGCCGC	AGCGGCCGTA	CCCAGGTGCT	GAAGAATTGA	CCCGGTTCTC	CGACCCGTAA
2000								

**FIG. 1A**

2/22

2001	AAAGCGCG	TTGCTGGCGT	TTTTCCATAG	GCTCCGCCCC	CCTGACGAGC	ATCACAAAA	TCGACGCTCA	AGTCAGAGGT	2080
2081	GGCGAAACCC	GACAGGACTA	TAAAGATACC	AGCGTTTCC	CCCTGGAAGC	TCCCTCGTGC	GCCTCCTGT	TCCGACCCTG	2160
2161	CCGCTTACCG	GATACCTGTC	CGCCTTCTC	CCTTCGGGAA	GCGTGGCGCT	TTCTCAATGC	TCACGCCTGA	GGTATCTCAG	2240
2241	TTCCGGTGTAG	GTGTTTCGCT	CCAAGCTGGG	CTGTGTGCAC	GAACCCCCCG	TTACGCCCGA	CCGCTGCCCC	TTATCCGGTA	2320
2321	ACTATCGTCT	TGAGTCCAAC	CCGGTAAGAC	ACGACTTATC	GCCACTGGCA	GCAGCCACTG	GTAACAGGAT	TAGCAGAGCG	2400
2401	AGGTATGTAG	GCGGTGCTAC	AGAGTTCTTG	AAGTGTGGC	CTAACTACGG	CTACACTAGA	AGGACAGTAT	TTGGTATCTG	2480
2481	CGCTCTGCTG	AAGCCAGTTA	CTTCGGGAA	AAGAGTTGGT	AGCTCTTGAT	CCGGCAAACA	AACCACCGCT	GGTAGCGGTG	2560
2561	GTTTTTTTGT	TTGCAAGCAG	CAGATTACGC	GCAGAAAAAA	AGGATCTCAA	GAAGATCCTT	TGATCTTTTC	TACGTGATCC	2640
2641	CGTAATGCTC	TGCCAGTGT	ACAACCAATT	AACCAATTCT	GATTAGAAA	ACTCATCGAG	CATCAAATGA	AACGTCAATT	2720
2721	TATTCATATC	AGGATTATCA	ATACCATATT	TTTGAAAAAG	CCGTTTCTGT	AATGAAGGAG	AAAACTCACC	GAGGCAGTTC	2800
2801	CATAGGATGG	CAAGATCCTG	GTATCGGCT	GCGATTCCGA	CTCGTCCAAC	ATCAATACAA	CCTATTAAAT	TCCCTCGTTC	2880
2881	AAAAATAAGG	TTATCAAGTG	AGAAATCACC	ATGAGTGACG	ACTGAATCCG	GTGAGAATGG	CAAAAGCTTA	TGCATTTCTT	2860
2961	TCCAGACTTG	TTCAACAGGC	CAGCCATTAC	GCTCGTCATC	AAAATCACTC	GCATCAACCA	AACCGTTATT	CATTGCTGAT	3040
3041	TGCGCCTGAG	CGAGACGAAA	TACGGGATCG	CTGTTAAAG	GACAATTACA	AACAGGAATC	GAATGCAACC	GGCGCAGGAA	3120
3121	CACTGCCAGC	GCAITCAACAA	TATTTTCACC	TGAATCAGGA	TATTCTTCTA	ATACCTGGAA	TGCTGTTTTTC	CCGGGGATCG	3200
3201	CAGTGGTGAG	TAACCATGCA	TCATCAGGAG	TACGGATAAA	ATGCTTGATG	GTGGAAGAG	GCATAAATTC	CGTCAGCCAG	3280
3281	TTTAGTCTGA	CCATCTCATC	TGTAACATCA	TTGGCAACGC	TACCTTTGCC	ATGTTTCAGA	AACAACCTCTG	GCGCATCGGG	3360
3361	CTTCCCATAC	AATCGATAGA	TTGTGCGACC	TGATTGCCCG	ACATTATCGC	GAGCCCATTT	ATACCCATAT	AAATCAGCAT	3440
3441	CCATGTTGGA	ATTTAATCGC	GGCCTCGAGC	AAGACGTTTC	CCGTTGAATA	TGGCTCATAA	CACCCCTTGT	ATTACTGTTT	3520
3521	ATGTAAGCAG	ACAGTTTTAT	TGTTCAATGAT	GATATATTTT	TATCTTGTGC	AATGTAACAT	CAGAGATTTT	GAGACACAAC	3600
3601	GTGGCTTTCC								3610

FIG.1B

3/22

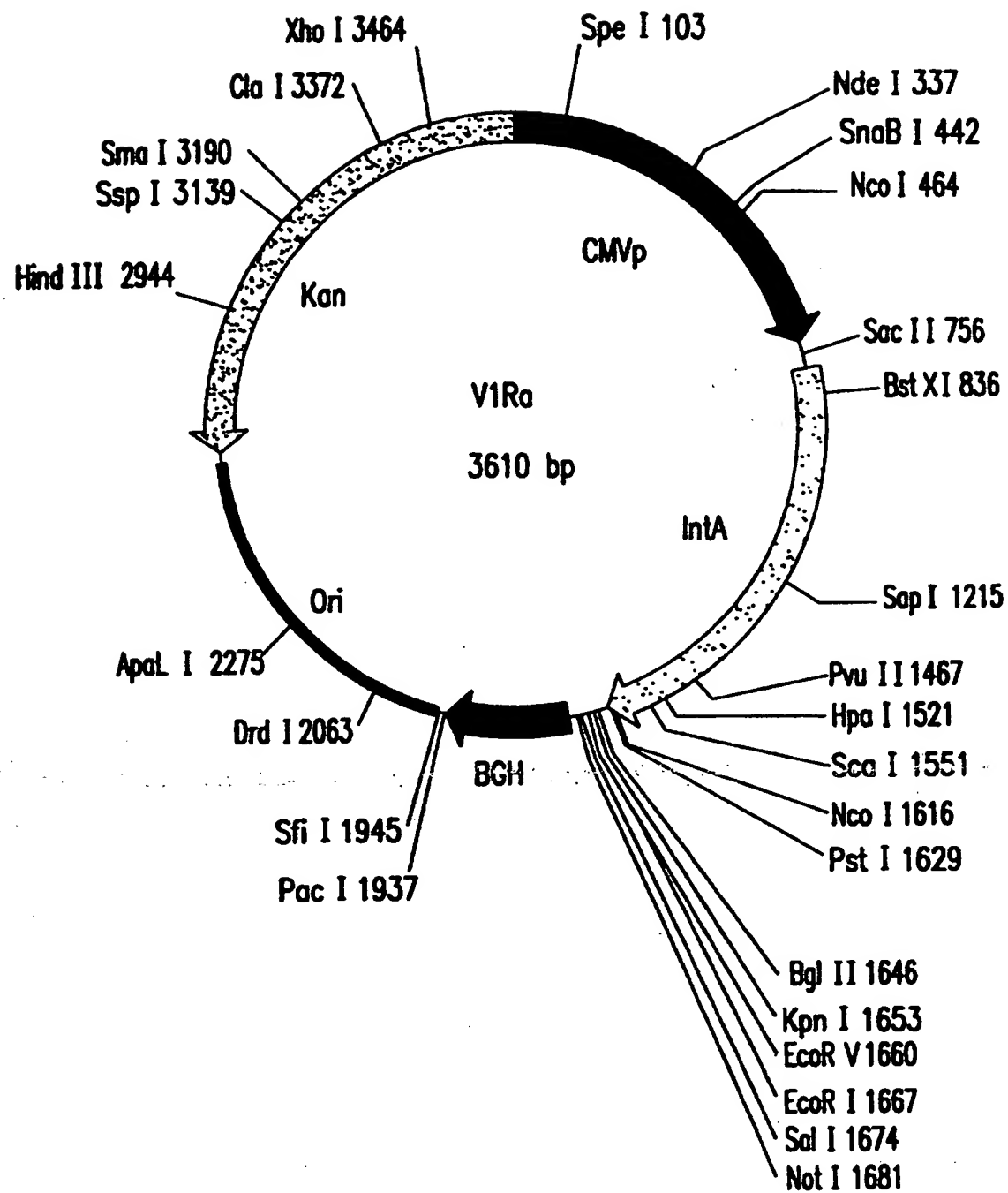


FIG.2

4/22

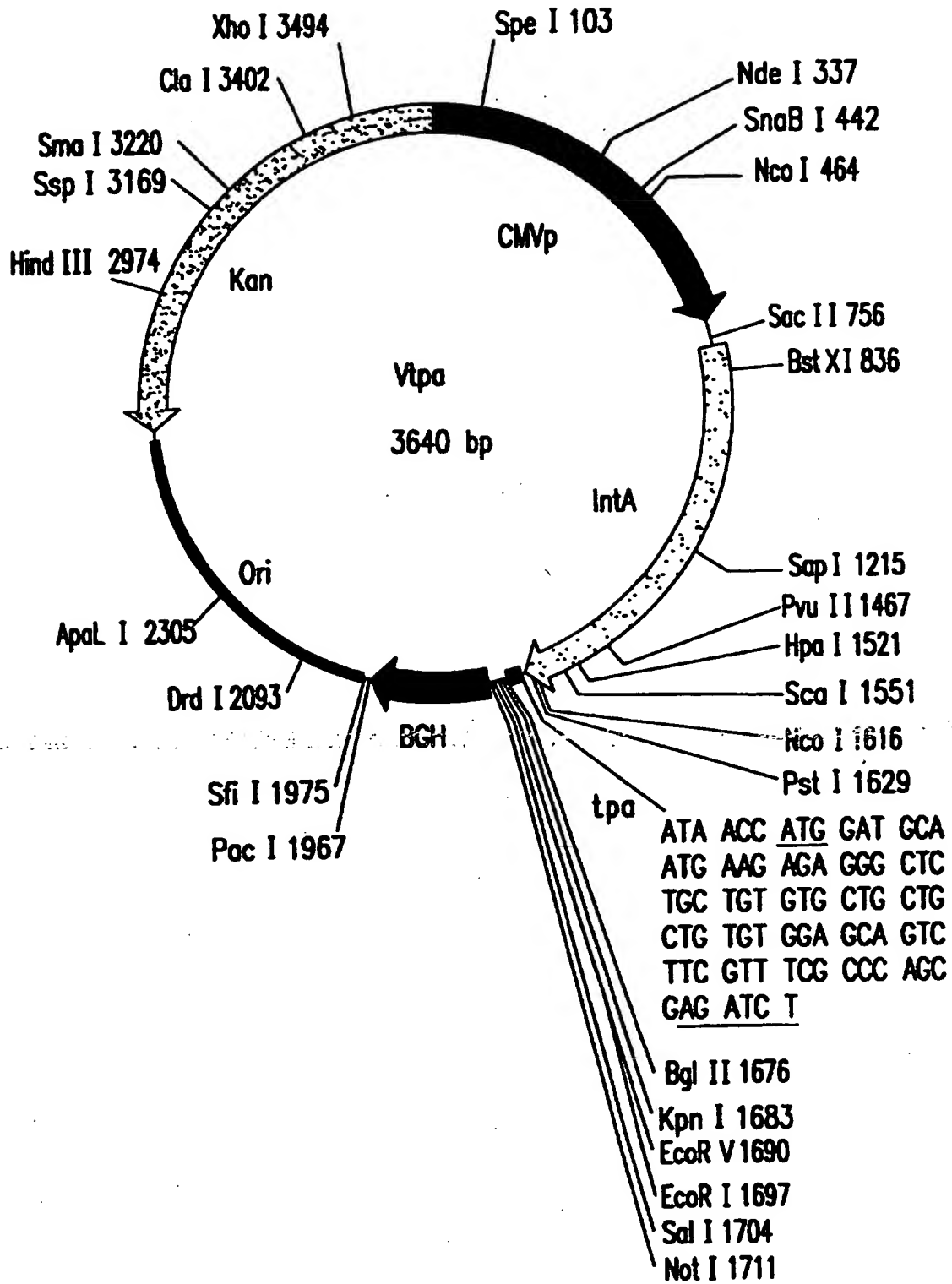


FIG.3

5/22

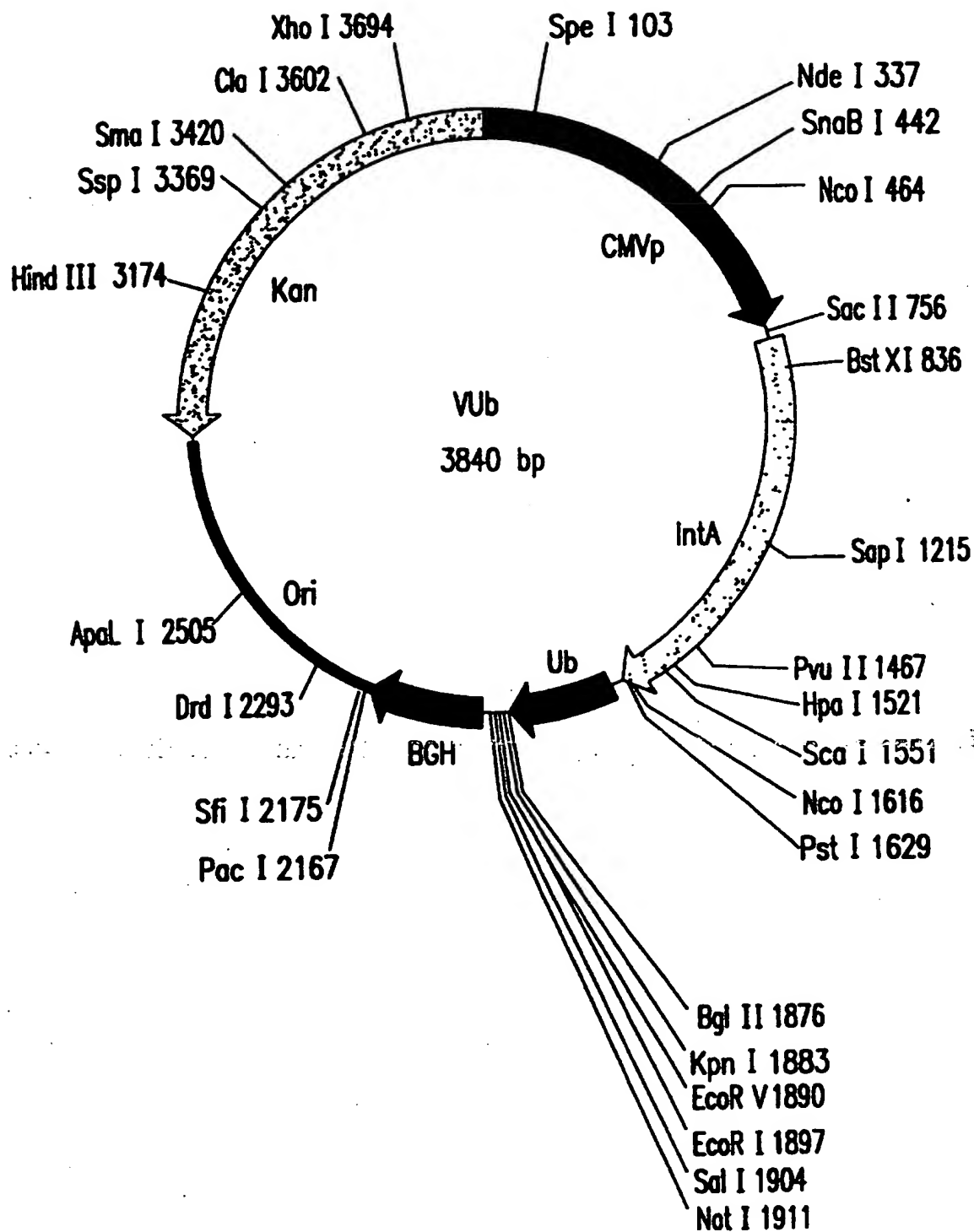


FIG.4

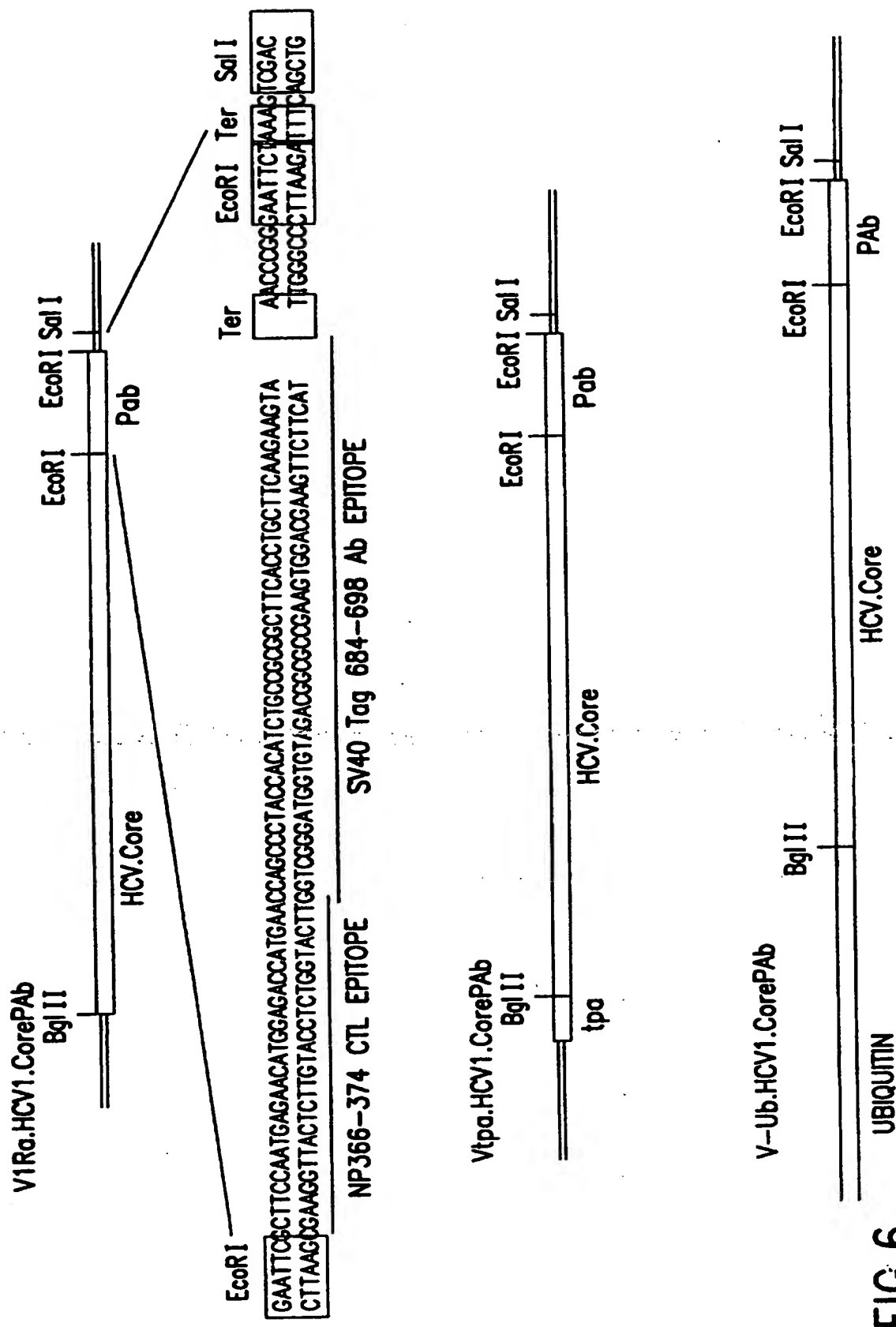


6/22

1/1 31/11  
 ATG AGC ACC AAC CCc AAg CCc CAg AGg ACC AAg aGg AAC ACC AAC aGg aGg CCcCAG  
 Met ser thr asn pro lys pro gln arg lys thr lys arg asn thr asn arg arg pro gln  
 61/21 91/31  
 GAT GTg AAG TTC Cct GGg GGa GGc CAG ATt GTg GGa GGg GTc TAC cTg cTg CCc aGg AGG  
 asp val lys phe pro gly gly gly gln ile val gly gly val tyr leu leu pro arg arg  
 121/41 151/51  
 GGC CCC AGG cTg GGg GTg aGg Gct Acc aGg AAG ACC Tct GAG aGg TCc Cag CCC aGg GGC  
 gly pro arg leu gly val arg ala thr arg lys thr ser glu arg ser gln pro arg gly  
 181/61 211/71  
 AGG aGg CAG CCC ATC CCC AAG GCC aGg aGg Cct GAG GGC cGc TCC TGG GcC CAG Cct GGC  
 arg arg gln pro ile pro lys ala arg arg pro glu gly arg ser trp ala gln pro gly  
 241/81 271/81  
 TAC CCC TGG CCC CTg TAT GGC AAT GAa GGC TTT GGC TGG Gct GGC TGG CTg CTg TCC CCC  
 try pro trp pro leu tyr gly asn glu gly phe gly trp ala gly trp leu leu ser pro  
 301/101 331/111  
 aGg GGC TCc aGg CCC tcc TGG GGC CCC ACa GAC CCC aGg aGg aGg TCc aGg AAC cTg GGC  
 arg gly ser arg pro ser trp gly pro thr asp pro arg arg arg ser arg asn leu gly  
 361/121 391/131  
 AAg GTg ATt GAC ACC CTg ACC Tgt GGC TTT Gct GAC CTg ATG GGC TAC ATC CCC CTg GTg  
 lys val ile asp thr leu thr cys gly phe ala asp leu met gly tyr ile pro leu val  
 421/141 451/151  
 GGg Gct Cct GTg GGa GGg GTg Gct AGG Gct CTg Gct CAT GGg GTg AGG GTg CTg GAG GAT  
 gly ala pro val gly gly val ala arg ala leu ala his gly val arg val leu glu asp  
 481/161 511/171  
 GGG GTg AAC TAT Gct ACT GGc AAC cTg Cct GGc TGC TCC TTC TCC ATC TTC CTg CTg GCC  
 gly val asn tyr ala thr gly asn leu pro gly cys ser phe ser ile phe leu leu ala  
 541/181 571/191  
 CTG CTc TCC TGC CTg ACa GTg Cct GCT TCT GCC  
leu leu ser cys leu thr val pro ala ser ala

FIG. 5

7/22



**FIG. 6**

8/22

1/1 31/11  
 ATG AGC ACG AAT CCT AAA CCT CAA AGA AAA ACC AAA CGT AAC ACC AAC CGC CGC CCA CAG  
 Met ser thr asn pro lys pro gln arg lys thr lys arg asn thr asn arg arg pro gln  
 61/21 91/31  
 GAC GTC Aag TTC CCg GGC GGT CAG ATC GTT GGT GGA GTT TAC TTC TTG CCG CGC AGG  
 asp val lys phe pro gly gly gln ile val gly gly val tyr leu leu pro arg arg  
 121/41 151/51  
 GGC CCC AGG TTG GGT GTG CGC GCG ACT aGG AAG ACT TCc GAG CGG TCG CAA CCT CGT GGa  
 gly pro arg leu gly val arg ala thr arg lys thr ser glu arg ser gln pro arg gly  
 181/61 211/71  
 AGG CGa CAG CCT ATC CCC AAG GCT CGc CGG CCC GAG GGC AGG TCC TGG GCT CAG CCC GGG  
 arg arg gln pro ile pro lys ala arg arg pro glu gly arg ser trp ala gln pro gly  
 241/81 271/91  
 TAC CCT TGG CCc CTc TAT GGC AAT GAG GGC Ttc GGG TGG GCA GGa TGG CTC CTG TCC CCC  
 tyr pro trp pro leu tyr gly asn glu gly phe gly trp ala gly trp leu leu ser pro  
 301/101 331/111  
 CGC GGC TCT CGg CCT agT TGG GGC CCC Act GAC CCC CGG CGT AGG TCG CGC AAT TTG GGT  
 arg gly ser arg pro ser trp gly pro thr asp pro arg arg ser arg asn leu gly  
 361/121 391/131  
 AAG GTC ATC GAT ACC CTC ACG TGC GGC TTC GCC GAC CTC ATG Gg TAC ATC CCG CTC GTC  
 lys val ile asp thr leu thr cys gly phe ala asp leu met gly tyr ile pro leu val  
 421/141 451/151  
 GGC GCC CCC GTA Gg GGC GTC GCC AGg GCC CTG GCG CAT GGC GTC AGG GtT cTG GAG GAC  
 gly ala pro val gly gly val ala arg ala leu ala his gly val arg val leu glu asp  
 481/161 511/171  
 GGG gtg AAC TAT GCA ACA GGG Aat tTg cCC GGT TGC TCT TTC TCT ATC TTC CTC cTG Gct  
 glu val asn tyr ala thr gly asn leu pro gly cys ser phe ser ile phe leu leu ala  
 541/181 571/191  
 CTg CTg TcG TGC CTG ACC GTC CCA Gct TCT GCT  
 leu leu ser cys leu thr val pro ala ser ala

FIG. 7

9/22

TABLE 3  
CODON UTILIZATION IN HUMAN PROTEIN-CODING SEQUENCES

a	b	c	d	e	f	a	b	c	d	e	f
F	UUU	68	0.35	193	4.5	Y	UAU	72	0.47	153	3.6
	UUC	125	0.65				UAC	81	0.53		
L	UUA	20	0.05	445	10.4	H	CAU	44	0.42	105	2.5
	UUG	42	0.09				CAC	61	0.58		
	CUU	50	0.11			Q	CAA	50	0.26	192	4.5
	CUC	99	0.22				CAG	142	0.74		
	CUA	30	0.07			N	AAU	51	0.34	148	3.5
	CUG	204	0.46				AAC	97	0.66		
I	AUU	28	0.23	123	2.9	K	AAA	137	0.45	303	7.0
	AUC	79	0.64				AAG	166	0.55		
	AUA	16	0.13			D	GAU	79	0.38	209	4.9
M	AUG	77	1.00	77	1.8		GAC	130	0.62		
V	GUU	35	0.13	266	6.2	E	GAA	125	0.40	311	7.3
	GUC	72	0.27				GAG	186	0.60		
	GUA	25	0.09			C	UGU	44	0.30	147	3.4
	GUG	134	0.50				UGC	103	0.70		
S	UCU	59	0.17	349	8.1	W	UGG	56	1.00	56	1.3
	UCC	91	0.26			R	CGU	19	0.09	215	5.0
	UCA	37	0.11				CGC	40	0.19		
	UCG	25	0.07				CGA	22	0.10		
	AGU	37	0.11				CGG	33	0.15		
	AGC	100	0.29				AGA	51	0.24		
P	CCU	51	0.24	212	4.9		AGG	50	0.23		
	CCC	86	0.41			G	GGU	36	0.15	245	5.7
	CCA	51	0.24				GGC	108	0.44		
	CCG	24	0.11				GGA	42	0.17		
T	ACU	47	0.20	238	5.6		GGG	59	0.24		
	ACC	113	0.47								
	ACA	50	0.21								
	ACG	28	0.12								
A	GCU	91	0.31	298	7.0						
	GCC	119	0.40								
	GCA	51	0.17								
	GCG	37	0.12								

TOTAL 4285 RESIDUES EXCLUDING  
N-TERMINAL METHIONINE RESIDUES

FIG.8

10/22

1/1 atg TAT GAG GTG aGg AAT GTc Tct GGc GTc TAC CAT GTg ACC AAt GAC TGc TCC AAC TCC  
 M Y E V R N V S G V Y H V T N D C S N S  
 31/11  
 61/21 tGc ATT GTc TAT GAG Gct Gct GAC ATG ATc ATG CAC ACC CCT GGc Tgt GTg CCa Tgt GTg  
 C I V Y E A A D M I M H T P G C V P C V  
 91/31  
 121/41 aGg GAG GGc AAC TCC TCC aGg TGC TGG GTg GCC CTg ACC CCC ACC CTg Gct GCC AGG AAC  
 R E G N S S R C W V A L T P T L A A R N  
 151/71  
 181/61 tcC tcC ATC CCC ACC ACC ATc aGg aGg CAT GTg GAC cTG CTg GTg GGc Gct GCT GCC  
 S S I P T T T I R R H V D L L V G A A A  
 211/71  
 241/81 CTg TGC Tct GcC ATG TAT GTG GGc GAC CTg TGT GGc TCT GTc TTC CTg GTg TCC CAG gTG  
 L C S A M Y V G D L C G S V F L V S Q L  
 331/111  
 301/101 TTC ACC TTC Tcc Ccc aGg aGg TAT GAG Act GTg CAG GAC TGC AAC TGC TCC CTg TAC CCT  
 F T S P R R Y E T V Q D C N C S L Y P  
 391/131  
 361/121 GGC CAT GTc Tct GGc CAC aGg ATG GCC TGG GAC ATG ATG ATG AAC TGG TCC CCC ACC Act  
 G H V S G H R M A W D M M M N W S P T T  
 451/151  
 421/141 GCC cTg GTG GTc Tcc CAG cTg CTg aGg ATt Ccc CAG Gct GTg GTG GAC ATG GTG TGT GGG  
 A L V V S Q L L R I P Q A V V D M V V G  
 511/171  
 481/161 GCC CAC TGG GGc GTg CTG Gct GGc CTg GCC TAC TAC TCC ATG GTG GGc AAC TGG GCC AAG  
 A H W G V L A G L A Y Y S M V G N W A K  
 571/191  
 541/181 GTg cTg ATT GTG ATG CTg CTg TTT Gct GGc GTg GAT GGc taa  
 V L I V M L L F A G V D G \*

FIG. 9

11/22

1/1  
 atg ACC ACC TAT GTc Tct GTG Ggc CAT Gcc tcC CAG ACC ACC aGg aGg GTg Gcc TCC TTC  
 M T Y V S V G H A S Q T T R R V A S F  
 31/11  
 61/21  
 TTC tcc CCT GGC Tct Gcc CAG AAg ATC CAg CTg GTg AAC ACC AAt GGC tcc TGG CAC ATC  
 F S P G S A Q K I Q L V N T N G S W H I  
 51/31  
 121/41  
 AAC AGG ACT GCC CTG AAt TGC AAt GAG TCC ATC AAC ATC Ggc TTC TTT Gct Gcc CTG TTC  
 N R T A L N C N E S I N T G F A A L F  
 151/51  
 181/61  
 TAT GTg AAG AAG TTC AAC Tcc TCT Ggc TGC Tct GAG aGg ATG Gcc tct TGc aGg CCC ATT  
 Y V K K F N S S G C S E R M A S C R P I  
 211/71  
 241/81  
 GAC AGG TTt GCC CAG GGC TGG Ggc CCC ATC ACC CAT GCT GAG Tcc aGg tcc Tct GAC CAG  
 D R F A Q G W G P I T H A E S R S S D Q  
 331/111  
 301/101  
 AGG CCa TAC TGC TGG CAC TAT Gcc CCc CAG CCa TGT Ggc ATt GTG Cct Gcc cTG CAT GTc  
 R P Y C W H Y A P Q P C G I V P A L H V  
 391/131  
 361/121  
 Tgt Ggc Cct GTc TAC TGc TTC ACC CCa tcc CCT GTg GTg Ggc ACg Act GAC aGg TTt  
 C G P V Y C F T P S P V V V G T T D R F  
 451/151  
 421/141  
 GGC GTg CCC ACC TAC AAC TGG Ggc GAC AAt GAG Act GAT GTG CTg CTg CTG AAC AAC ACC  
 G V P T Y N W G D N E T D V L L L N N T  
 511/171  
 481/161  
 aGg CCc CCc CAg Ggc AAC TGG TTt Ggc TGc Acc TGG ATG AAC tcc Act Ggc TTC ACC AAG  
 R P P Q G N W F G C T W M N S T G F T K

FIG.10A

12/22

541/181 571/191  
 Acc Tgt Ggc Ggc Ccc Cca Tgc Aac Att Ggc Ggc Gct Ggc Aac Aac Acc cTg Acc Tgc Ccc  
 T C G G P P C N I G G A G N N T L T C P  
 601/201 631/211  
 Act Gac Tgc Ttc aGg Aag Cat Cct GAG GCC Acc TAC ACC AAG Tgt Ggc Tct Ggc Cca Tgg  
 T D C F R K H P E A T Y T K C G S G P W  
 661/221 691/231  
 cTg Acc Ccc AGg Tgc ATg GTg Gac Tac Cca Tac Agg CTg Tgg CAC TAC Cca Tgc Acc TTC  
 L T P R C M V D Y P Y R L W H Y P C T F  
 721/241 751/251  
 AAC TTC ACC ATC TTC AAG ATC AGG ATG TAT GTG Ggc Ggc GTG GAG CAC AGG CTg Aat Gct  
 N F T I F K I R M Y V G G V E H R L N A  
 781/261 811/271  
 Gcc Tgc AAC Tgg Acc aGg Ggc GAG aGg Tgc AAC Att GAG GAC AGG GAC AGG Tct GAG CTg  
 A C N W T R G E R C N I E D R D R S E L  
 841/281 871/291  
 tcc Ccc CTg CTg CTg Tcc Acc Act GAG Tgg Cag ATc CTg Cca Tgc Tcc Ttc Acc Acc CTg  
 S P L L L S T T E W Q I L P C S F T T L  
 901/301 931/311  
 Cct Gcc CTg Tcc Act Ggc cTg ATc Cat Cag AAC Att GTg Gat GTg Cag TAC CTg  
 P A L S T G L I H L H Q N I V D V Q Y L  
 961/321 991/331  
 Tat Ggc GTg Ggc Tct Gct GTg GTc Tcc Att GTg ATc Aag Tgg GAG Tat GTg CTg CTg CTg  
 Y G V G S A V V S I V I K W E Y V L L L  
 1021/341  
 TTC CTg CTg CTg Gct Gat Gcc taa  
 F L L L A D A \*

FIG.10B

13/22

1/1 atg TAT GAG GTG aGg AAT GTc TcT GGc GTc TAC CAT GTg ACC AAT GAC TGC TCC AAC TCC  
 M Y E V R N V S G V Y H V T N D C S N S  
 31/11  
 61/21 tGc ATT GTc TAT GAG GcT GcT GAC ATG ATc ATG CAC ACC CcT GGc Tgt GTg CCa Tgt GTg  
 C I V Y E A A D M I M H T P G C V P C V  
 91/31  
 121/41 aGg GAG GGc AAC TCC TCC aGg TGC TGG GTg GGc CTg ACC CCC ACC CTg GcT GCC AGG AAC  
 R E G N S S R C W V A L T P T L A A R N  
 151/51  
 181/61 tcC tcC ATC CCC ACC ACC ATc aGg aGg CAT GTg GAC cTG CTg GTg GGc GcT GcT GCC  
 S S I P T T I R R H V D L L V G A A A  
 211/71  
 241/81 CTg TGC TcT GcC ATG TAT GTG GGc GAC CTg TGT GGc TCT GTc TTC CTg GTg TCC CAG cTG  
 L C S A M Y V G D L C G S V F L V S Q L  
 271/91  
 301/101 TTC ACC TTC TCC CCc aGg aGg TAT GAG ACT GTg CAG GAC TGC AAC TGC TCC CTg TAC CcT  
 F T F S P R R Y E T V Q D C N C S L Y P  
 331/111  
 361/121 GGC CAT GTc TcT GGc CAC aGg ATG GcC TGG GAC ATG ATG ATG AAC TGG TCC CCC ACC ACT  
 G H V S G H R M A W D M M M N W S P T T  
 391/131  
 421/141 GCC cTG GTG GTc TCC CAG cTG CTg aGg ATt CCc CAG GcT GTg GTG GAC ATG GTG GTG GGC  
 A L V V S Q L L R I P Q A V V D M V V G  
 451/151  
 481/161 GCC CAC TGG GGc GTg CTG GcT GGc CTg GCC TAC TAC TCC ATG GTG GGc AAC TGG GCC AAG  
 A H W G V L A G L A Y Y S M V G N W A K  
 511/171

FIG.11A



14/22

541/181 571/191  
 GTg cTg ATT GTG ATG CTg CTg TTT Gct GGC GTg GAt GGC ACC ACC TAt GTc Tct GTG GGC  
 V L I V M L L F A G V D G T T Y V S V G  
 601/201 631/211  
 CAT Gcc tcC CAG ACC ACC aGg aGg GTg Gcc TCC TTC TTC tcc CCT GGC Tct GCC CAG AAG  
 H A S Q T T R R V A S F F S P G S A Q K  
 661/221 691/231  
 ATC CAg CTg GTg AAC ACC AAt GGC tcc TGG CAC ATC AAC AGG ACT GCC CTG AAt TGC AAt  
 I Q L V N T N G S W H I N R T A L N C N  
 721/241 751/251  
 GAG TCC ATC AAC ACT GGC TTC TTT Gct Gcc CTG TTC TAt GTg AAG AAG TTC AAC Tcc TCT  
 E S I N T G F F A A L F Y V K K F N S S  
 781/261 811/271  
 GGC TGC Tct GAG aGg ATG Gcc tct Tgc aGg CCC ATT GAC AGG TTt GCC CAG GGC TGG GGC  
 G C S E R M A S C R P I D R F A Q G W G  
 841/281 871/291  
 CCC ATC ACC CAT GCT GAG Tcc aGg tcC Tct GAC CAG AGG CCa TAC TGC TGG CAC TAt GCC  
 P I T H A E S R S S D Q R P Y C W H Y A  
 901/301 931/311  
 CCC CAg CCa TGT GGC ATt GTG Cct Gcc cTg CAT GTc Tgt GGC Cct GTc TAc Tgc TTC ACC  
 P Q P C G I V P A L H V C G P V Y C F T  
 961/321 991/331  
 CCa tcC CCT GTg GTg GGC ACC Act GAC aGg TTt GGC GTg CCC Acc TAc AAC TGG GGC  
 P S P V V V G T T D R F G V P T Y N W G  
 1021/341 1051/351  
 GAC AAT GAG Act GAt GTG CTg CTg AAC AAC ACC aGg CCC CCC CAG GGC AAC TGG TTe  
 D N E T D V L L L N N T R P P Q G N W F

FIG.11B

15/22

1081/361  
 GGC Tgc Acc TGG ATG AAC tcc Act Ggc Ttc Acc AAG Acc Tgt Ggc CCC Cca Tgc AAC  
 G C T W M N S T G F T K T C G G P P C N  
 1111/37  
 1141/381  
 ATT Ggc Ggc Gct GGC AAC AAC ACC cTg ACC Tgc CCC Act Gac Tgc TTC aGg AAG Cat Cct  
 I G G A G N N T L T C P T D C F R K H P  
 1171/391  
 1201/401  
 GAG GCC Acc TAC ACC AAG Tgt Ggc Tct Ggc Cca Tgg cTg Acc CCC AGG Tgc ATg GTg GAC  
 E A T Y T K C G S G P W L T P R C M V D  
 1231/411  
 1261/421  
 TAC Cca TAC AGg CTg TGG CAC TAC Cca Tgc Acc TTC AAC Ttc Acc ATC Ttc AAG ATC AGG  
 Y P Y R L W H Y P C T F N F T I F K I R  
 1291/431  
 1321/441  
 ATG TAT GTg Ggc Ggc GTg GAG CAC AGG CTg Aat Gct Gcc Tgc AAC Tgg Acc aGg Ggc GAG  
 M Y V G G V E H R L N A A C N W T R G E  
 1351/451  
 1381/461  
 aGg Tgc AAC ATg GAG CAC AGG Gac AGG Tct GAG CTg tcc Ccc CTg CTg Tcc Acc Act  
 R C N I E D R D R S E L S P L L L S T T  
 1411/471  
 1441/481  
 GAG Tgg CAG ATc CTg Cca Tgc Tcc Ttc Acc Acc CTg Cct Gcc CTg Tcc ACT Ggc cTg ATC  
 E W Q I L P C S F T T L P A L S T G L I  
 1471/491  
 1501/501  
 CAT CTg CAT CAG AAC ATT GTg Gat GTg Cag TAC CTg TAt GGc GTg Ggc Tct Gct GTg GTc  
 H L H Q N I V D V Q Y L Y G V G S A V V  
 1531/511  
 1561/521  
 TCC ATT GTg ATC Aag TGG GAG TAt GTg CTg CTg TTC CTg CTg CTg Gct Gat Gcc taa  
 S I V I K W E Y V L L L F L L L A D A \*

FIG.11C

16/22

1/1 atg Tct Ggc Tcc Tgg CTg AGG GAT GTc TGG GAC TGG ATc TGC Act GTG CTG ACT GAC TCC  
 M S G S W L R D V W D W I C T V L T D F  
 31/11  
 61/21 AAG ACC TGG CTg CAT TCC AAG CTg CTG CCC aGG CTG CCT GGC GAC CCa TTC TTC TCC TGC  
 K T W L H S K L L P R L P G D P F F S C  
 91/31  
 121/41 TAC AGG GGC GTc TGG aGG GGC GAT GGC GTg ATG CAg ACC ACC TGC CCa TGT  
 Cag aGg Ggc Tgc Tgg aGg Ggc GAT Ggc GTg ATg Cag ACC ACC TGC CCa TGT  
 Q R G Y R G V W R G D G V M Q T T C P C  
 151/51  
 181/61 GGC GCC CAG ATC ACT GGC CAT GTg AAG AAT GGC TCC ATG AGG ATt GTg GGC CCc AAg ACC  
 G A Q I T G H V K N G S M R I V G P K T  
 211/71  
 241/81 Tgc tcc AAC Acc TGG CAT GGC Acc TTC CCC ATC AAT GCC TAC ACC ACT GGC CCa TGC ACC  
 C S N T W H G T F P I N A Y T T G P C T  
 301/101 Cca TCC CCT GCC CCC AAC TAC TCC AGG GGC CTG TGG aG GTG GCT GCT GAG GAG TAT GTG  
 P S P A P N Y S R A L W R V A A E Y V V  
 331/111  
 361/121 Acc aGG GTG GGC GAC TTC CAC TAT GTG ACT GGC ATG ACC ACT GAC AAT GTg AAG  
 GAG GTg Acc aGg GTg Ggc Gac Ttc Cac Tat GTg Act Ggc ATg Acc Act Gac Aat GTg Aag  
 E V T R V G D F H Y V T G M T T D N V K  
 391/131  
 421/141 TGC CCa TGC CAG GTg CCT GCC CCT GAG TTC TTC ACT GAG GTG GAT GGC GTG aGG CTG CAC  
 C P C Q V P A P E F T E V D G V R L H  
 451/151  
 481/161 AGG TAT GCC CCT GGC TGC AAg CCc CTg CTg aGG GAT GAG GTg ACC TTC CAG GTg GGC CTg  
 R Y A P A C K P L L R D E V T F Q V G L  
 511/171

FIG.12A

17/22

541/181  
 AAC CAG TTC Cct GTg Ggc Tcc CAG CTg CCa TGT GAG Cct GAg Cct GAT GTg Act GTG CTg  
 N Q F P V G S Q L P C E P E P D V T V L  
 571/191  
 601/201  
 ACC TCC ATG CTg Act GAg CCa TCC CAC A:c Act Gct GAG Act Gcc AAG aGg AGG cTG GCC  
 T S M L T E P S H I T A E T A K R R L A  
 631/211  
 661/221  
 AGg GGC TCC Cct CCa TCC CTG GCC tcC TCC TGcc tcC CAG CTg TCT Gct CCa Tcc cTG  
 R G S P P S L A S S S A S Q L S A P S L  
 691/231  
 721/241  
 AAG GCC ACC TGC ACC ACC aGg CAT GAC TCC Cct GAT Gct GAC CTg ATt GAG GCC AAC CTg  
 K A T C T T R H D S P D A D L I E A N L  
 751/251  
 781/261  
 CTG TGG aGg CAG GAG ATG GGC Ggc AAC ATC ACC aGg GTG GAG Tct GAG AAC AAG GTg GTg  
 L W R Q E M G G N I T R V E S E N K V V  
 811/271  
 841/281  
 ATc CTg GAC Tcc TTT GAg CCC CTg aGg Gct GAG GAG GAT GAG AGG GAg GTc Tct GTG Gct  
 I L D S F E P L R A E E D E R E V S V A  
 901/301  
 Gct GAG ATC CTg aGg AAg tcc AGG AAg TTC CCC Cct GCC cTG CCC ATc TGG GCg aGg CCa  
 A E I L R K S R K F P P A L P I W A R P  
 931/311  
 961/321  
 tcc TAC AAC CCa CCC CTg CTg GAG TCC TGG AAG GAC Cct GAC TAT GTg CCC Cct GTG GTg  
 S Y N P P L L E S W K D P D Y V P P V V  
 991/331  
 1021/381  
 CAT Ggc TGC CCC CTG CCC ACC ATG CCC CCa CCC ATc CCC CCa CCC aGg AGG AAG AGG  
 H G C P L P P T M A P P I P P P R R K R  
 1051/371

FIG.12B

18/22

1081/361 1111/371  
 Act GTg GTg CTg Act GAg TCC Act GTc Tcc TCT GCC cTG GCT GAG CTg GCC Acc AAG ACC  
 T V V L T E S T V S S A L A E L A T K T  
 1141/381 1171/391  
 TTC GGC tcC Tct TCC Tct GCT GTg GAC tct GGC Act GCc ACG GCC CCc CCT GAC CAG  
 F G S S G S S A V D S G T A T A P P D Q  
 1201/401 1231/411  
 CCa Tct GAT GAT GGC GAC AGg GGc Tct GAT GAT GAG TCC TAC TCC TCC ATG CCC CCC CTg  
 P S D D G D R G S D D E S Y S S M P P L  
 1261/421 1291/431  
 GAG GGC GAG CCT GGC GAC CCT GAC CTg tct GAT GGc Tcc TGG TCC Act GTc tct GAG GAG  
 E G E P G D P D L S D G S W S T V S E E  
 1321/441  
 GCC tct GAG GAT GTg GCc TGC TGC TCC taa  
 A S E D V A C C S \*

FIG.12C

19/22

1/1  
 ATG TCC TAC ACC TGG ACT GGC GCC CTg ATC ACC CCa Tgt Gct Gct GAG GAG tcc AAG CTG  
 M S Y T W T G A L I T P C A A E E S K L  
 31/11  
 61/21  
 CCC ATC AAC CCC CTG tcc AAC TCC CTG CTG agg CAT CAC AAC ATG GTc TAT GCC ACC ACC  
 P I N P L S N S L L R H H N M V Y A T T  
 91/31  
 121/41  
 TCC agg tct Gct GGC CTg agg CAG AAG AAG GTg ACC TTT GAC AGg CTG CAT GTg CCT GAT  
 S R S A G L R Q K K V T F D R L H V P C  
 151/51  
 181/61  
 GAC CAC TAC agg GAT GTG CTg AAG GAG ATG AAG GCC AAG GCC TCC ACT GTg AAG GCg AAG  
 D H Y R D V L K E M K A K A S T V K A K  
 211/71  
 241/81  
 CTg CTg TCT GTg GAg GAg GCC TGC AAG CTG ACC CCT CCC CAC Tct GCC AGg TCC AAG TTT  
 L L S V E E A C K L T P P H S A R S K F  
 331/111  
 301/101  
 GGC TAT GGC GCC AAG GAT GTg agg AAC CTg TCC tcc AAG Gct GTg AAC CAC ATC CAC Tct  
 G Y G A K D V R N L S S K A V N H I H S  
 391/131  
 361/121  
 GTc TGG AAG GAC CTG CTG GAg GAC ACT GAg ACC CCC ATT GAC ACC ACC ATC ATG GCC AAG  
 V W K D L L E D T E T P I D T T I M A K  
 451/151  
 421/141  
 AAT GAG GTc TTC TGT GTg CAg CCT GAG AAG GGC GGC agg AAG CCT GCC agg CTg Att GTc  
 N E V F C V Q P E K G G R K P A R L I V  
 511/171  
 481/161  
 TTC CCT GAg CTg Ggc GTg agg GTG Tgt GAG AAg ATG GCC CTg TAT GAT GTG GTc TCC ACC  
 F P E L G V R V C E K M A L Y D V V S T

FIG. 13A

20/22

541/181 CAG Gct GTG ATG GGC TCC TCC TAT Ggc TTC CAG TAC TCC CCT Ggc CAG aGG GTg  
 CTg CCc L P Q A V M G S S Y G F Q Y S P G Q R V  
 571/191  
 601/201 GAG TTC CTG GTG AAT GCC TGG AAg TCC AAG AAg AAC CCc ATG GGC TTt Gcc TAc TGC ACC  
 E F L V N A W K S K K N P M G F A Y C T  
 631/211  
 661/221 TTT GAC TCC Act GTg ACT GAG tct GAC ATC aGg GTg GAG GAG TCC ATc TAC CAg  
 aGg TGc R C F D S T V T E S D I R V E E S I Y Q  
 691/231  
 721/241 TGC TGT GAC cTG Gct CCT GAG GCC AGg CAG GTg ATc AGG TCC CTg ACT GAG aGG CTg TAc  
 C C D L A P E A R Q V I R S L T E R L Y  
 751/251  
 781/261 GGC CCC CTG Acc AAC TCC AAg Ggc CAG AAC Tgt Ggc TAc aGg aGG TGC aGg GCC  
 ATt GGC I G G P L T N S K G Q N C G Y R R C R A  
 811/271  
 841/281 tct GGC GTG CTG Acc ACT AAC Tgt Ggc AAc ACC CTg Acc TGc TAc cTG AAg GCC TCT Gct  
 S G V L T T N C G N T L T C Y L K A S A  
 871/291  
 901/301 Gct TGc aGg GCT GCC AAg CTg CAT GAC TGC Acc ATG CTg GTc Tgt Ggc Gat GAC CTg GTg  
 A C R A A K L H D C T M L V C G D D L V  
 931/311  
 961/321 GTg ATC TGT GAg tct Gct Ggc Acc CAG GAG Gat Gct Ggc tcc CTg aGg GTc TTC ACT GAG  
 V I C E S A G T Q E D A A S L R V F T E  
 991/331  
 1021/341 GCC ATG Acc AGG TAc TCT GCC CCc CCT Ggc GAC CCt CCC CAg CCt GAg TAt GAC cTG GAG  
 A M T R Y S A P P G D P P Q P E Y D L E  
 1051/351

FIG.13B

21/22

1081/361  
 cTg ATc Acc Tcc Tgc Tcc Tcc Aat Gtc Tct GTg GCC CAT Gat Gcc Tct GGC AAG aGg GTc  
 L I T S C S S N V S V A H D A S G K R V  
 1111/371  
 1141/381  
 TAC TAc CTg Acc aGg GAc CCC Acc ACC CCC CTg GCC AGG Gct Gcc TGG GAg Act Gcc AGg  
 Y Y L T R D P T T P L A R A A W E T A R  
 1171/391  
 1201/401  
 CAC Acc CCT GTg AAC TCC TGG CTg GGC AAC ATC ATC ATG TAT Gcc CCC Acc CTG TGG Gcc  
 H T P V N S W L G N I I M Y A P T L W A  
 1231/411  
 1261/421  
 AGG ATG ATc CTg ATG ATG Acc CAC TTC TTC TCC ATC CTg CTg Gcc CAG GAG CAG CTg GAg AAg  
 R M I L M T H F F S I L L A Q E Q L E K  
 1291/431  
 1321/441  
 GCC CTG Ggc Tgc CAG ATt TAT Ggc GCC Acc TAC TTC ATT GAg Ccc CTg GAC CTg Ccc CAG  
 A L G C Q I Y G A T Y F I E P L D L P Q  
 1351/451  
 1381/461  
 ATC ATc CAG aGg CTg CAT Ggc CTg tct Gcc TTC TCC CTg CAC tcc TAC Tcc Cct Ggc GAg  
 I I Q R L H G L S A F S L H S Y S P G E  
 1411/471  
 1441/481  
 ATC AAC AGG GTG Gcc Tcc Tgc CTg AGG AAg CTg Ggc GTg Ccc CCC cTG aGg GTg TGG AGg  
 I N R V A S C L R K L G V P P L R V W R  
 1471/491  
 1501/501  
 GAc aGg GCC AGg tct GTg aGg Gcc AAg CTg CTG TCC CAG Ggc AGG Gct GCC Acc TGT  
 H R A R S V R A K L L S Q G G R A A T C  
 1531/511  
 1561/521  
 GGC AAg TAc CTg TTC AAC TGG Gct GTG AGG ACC AAg CTg AAg CTg ACC CCC ATc Cct GCT  
 G K Y L F N W A V R T K L K L T P I P A

FIG.13C



22/22

1621/541  
 GCC TCC CAG cTG GAC cTg Tct GGC TGG TTt GTg GCT GGc TAC tct GGc GGc GAC ATc TAc  
 A S Q L D L S G W F V A G Y S G G D I Y  
 1681/561  
 CAC tcC CTG Tcc aGg GCC aGg CCC aGg TGG TTC ATG TGG TGC CTg CTg CTg TCT GTg  
 H S L S R A R P R W F M W C L L L S V  
 1741  
 GGc GTg GGC ATC TAC CTG CTg CCC AAC aGG TGA  
 G V G I Y L L P N R \*

1651/551

1711/571

1771/591

FIG.13D

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/09884

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A61N 43/04; C12Q 1/68; C12N 15/00; C07H 21/02; A61K 39/00

US CL : 514/44; 435/6, 320.1; 536/23.1; 434/184.1, 192.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/6, 320.1; 536/23.1; 434/184.1, 192.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, MEDLINE, BIOSIS, EMBASE, CAPLUS, WPIDS, SCISEARCH

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Selby et al. Expression, identification and subcellular localization of the proteins encoded by the hepatitis C viral genome. Journal of General Virology. 1993. Vol. 74, pages 1103-1113, see entire document.	1-3
X	Bukh et al. Sequence analysis of the core gene of 14 hepatitis C virus genotypes. Proc. Natl. Acad. Sci. August 1994. Vol. 91, pages 8239-8243, see entire document.	1-3
Y	Lathe. Synthetic Oligonucleotide Probes Deduced from Amino Acid Sequence Data Theoretical and Practical Considerations. J. Mol. Biol. 1985. Vol. 183, pages 1-12, see entire document.	1-3

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"A" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"T" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

28 AUGUST 1997

Date of mailing of the international search report

11 SEP 1997

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/09884

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Grantham et al. Codon catalog usage is a genome strategy modulated for gene expressivity. Nucleic Acids Research. 1981. Vol. 9, No. 1, pages r43-r74, see entire document.	1-3
A, P	Ide et al. Characterization of the nuclear localization signal and subcellular distribution of hepatitis C virus nonstructural protein NS5A. Gene. December 1996. Vol. 182, pages 203-211, see entire document.	1-3, 8-26
X	US 5,514,539 A (BUKH et al.) 07 May 1996, see entire document.	1-3, 8-26

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/09884

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☒ Claims Nos.: 4-7  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
  
The enclosed copy of claims 4, 5 were not legible and claims 6, 7 depend on those claims.
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.